THE SIGNIFICANCE OF CTLA-4 (CYTOTOXIC T-LYMPHOCYTE ASSOCIATED PROTEIN 4) GENE POLYMORPHISM IN SUSCEPTIBILITY TO SYSTEMIC LUPUS ERYTHEMATOSUS

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BONAFIDE CERTIFICATE

This to certify that this dissertation work entitled "**THE SIGNIFICANCE OF CTLA-4(CYTOTOXIC T-LYMPHOCYTE ASSOCIATED PROTEIN 4) GENE POLYMORPHISM IN SUSCEPTIBILITY TO SYSTEMIC LUPUS ERYTHEMATOSUS"** is the original bonafide work done by **Dr.M.DIVYA**, Post Graduate Student, Institute of Biochemistry, Madras Medical College, Chennai under our direct supervision and guidance.

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DECLARATION

I, **Dr.M.DIVYA** , Post Graduate , Institute of Biochemistry, Madras Medical College, solemnly declare that the dissertation titled "**THE SIGNIFICANCE OF CTLA-4(CYTOTOXIC T-LYMPHOCYTE ASSOCIATED PROTEIN 4) GENE POLYMORPHISM IN SUSCEPTIBILITY TO SYSTEMIC LUPUS ERYTHEMATOSUS"** is the bonafide work done by me at Institute of Biochemistry, Madras Medical College under the expert guidance and supervision of **Prof.Dr.K.PRAMILA,** M.D., Professor & Head, Institute of Child Health, Madras Medical College. The dissertation is submitted to the Tamil Nadu Dr.M.G.R Medical University towards partial fulfillment of requirement for the award of M.D., Degree (Branch XIII) in Biochemistry.

Place: Chennai

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CONTENTS

ABBREVIATION

1.	CTLA-4	-	Cytotoxic T Lymphocyte Antigen 4
	a) sCTLA-4	-	soluble CTLA4 protein
	b) (m)CTLA-4	-	membrane CTLA4 protein
2.	SLE	-	Systemic Lupus erythematosus
3.	a. ARMS PCR	-	Amplification Refractory mutation system
	b. T-ARMS PCR	-	Tetra primer ARMS PCR
4.	ACR	-	American college for Rheumatology
5.	BAFF	-	B-cell Activation factor
6.	BANK 1 gene	-	B Cell scaffold protein with ankyrin repeats
7.	BLK gene	-	B Lymphocyte specific tyrosine kinase
8.	CRP	-	C- reactive protein
9.	DNAMT1	-	DNA methyltransferase 1
10.	FCG2A/ FCG3A	-	Fc gamma receptor IIa / Fc gamma receptor IIIa
11.	GAD 45 alpha	-	Growth arrest and DNA damage induced 45 alpha.
12.	GWAS	-	Genome wide association study
13.	*ICOS*	-	Inducible Costimulator
14.	IRF 5	-	Interferon regulatory factor 5
15.	ISN/RPS	-	International Society of Nephrology / Renal Pathology Society
16.	ITGAM	-	Integrin αM
17.	LAT protein	-	Linker for activation of T cells
18.	LRR	-	Leucine rich repeats

19.	MDRD	-	Modification of diet in renal disease
20.	NPSLE	-	Neuropsychiatric systemic lupus erythematosus
21.	pDC	-	Plasmacytoid dendritic cells
22.	PDCD1	-	Programmed cell death 1
23.	PP2A	-	Protein Phosphatase 2A
24.	PTPN22	-	Protein tyrosine phosphatase, non-receptor type 22
25.	SEB	-	Staphylococcal enterotoxin B
26.	SHP-2	-	Src Homology Phosphatase 2
27.	SNP	-	Single nucleotide Polymorphism
28.	STAT 4	-	Signal transducer and activator of transcription factor 4
29.	TAE	-	Tris acetate EDTA buffer
30.	TIR	-	Toll/IL-1 receptor
31.	TLR	-	Toll Like Receptors
32.	TNFAIP3	-	Tumor necrosis factor- induced protein 3
33.	TNIP1	-	TNFAIP3 interacting protein 1
34.	Tregs cells	-	T regulatory cells
35.	TREX 1	-	3-prime repair exonuclease 1
36.	UTR	-	Untranslated region

THE SIGNIFICANCE OF CTLA-4(CYTOTOXIC T-LYMPHOCYTE ASSOCIATED PROTEIN 4) GENE POLYMORPHISM IN SUSCEPTIBILITY TO SYSTEMIC LUPUS ERYTHEMATOSUS

ABSTRACT

INTRODUCTION:

CTLA4 protein is expressed on the surface of activated T cells, exhibiting a negative regulatory role. This protein along with CD28, a positive regulator of T cells maintains immune system homeostasis. Polymorphism in CTLA4 gene leads to reduced surface expression of CTLA4 protein, resulting T cells become overactive against self-antigens, increasing the susceptibility of Systemic Lupus Erythematosus.

OBJECTIVE:

- To assess frequency of single nucleotide polymorphism in CTLA4 gene among cases/controls,
- To analyze effect of polymorphism over protein and disease expression

MATERIALS AND METHODS:

CTLA4 genotype (A/G) of 100 known SLE patients was compared with 100 matched healthy controls. Genotypic expression was correlated to level of CTLA4 protein and expression of disease. Genotype of patients &controls was detected by ARMS PCR, Level of protein measured by ELISA.

RESULTS:

The genotype distribution is in Hardy Weinberg equilibrium. (Chi square = 0.54 P =0.7). The frequency of CTLA4 +49 GG genotype is higher in cases than controls. The level of soluble fragment of CTLA-4 protein is significantly higher in cases compared to controls. Positive correlation exists between CTLA4 protein levels and GG genotype ($p < 0.001$). GG genotype individual have comparatively younger age of onset of disease.

CONCLUSION:

Transition from A to G allele at 49^{th} position in exon1 of CTLA4 gene will result in exchange of alanine for Threonine. There is altered intracellular trafficking, surface expression of the protein gets reduced and plasma level gets elevated, increasing susceptibility to SLE. Hence G allele in exon1 of CTLA4 gene will increase risk of SLE.

KEY WORDS:

Systemic lupus Erythematosus, Cytotoxic T Lymphocyte antigen 4, ARMS PCR, Single nucleotide polymorphism.

INTRODUCTION

Systemic lupus erythematosus is one of the most significant diseases in the field of medicine. The disease predominantly targets young women of reproductive age group and has the potential to cause significant physical disfigurement, extensive morbidity, with occasional mortality. Identification of immunological contributors to lupus had been the focus of intense research in the initial era. But the recent efforts, apart from supporting the central role of immune system in disease pathogenesis have also defined the genetic variations that underlie susceptibility to lupus. So the view on lupus pathology is extended beyond autoantibodies to include the contribution by several candidate genes. Single nucleotide polymorphisms play crucial role in the disease pathogenesis and progression. The recent advances concentrated on genetic variations have provided important insights into how the intersection of genetic variations and environmental triggers amplifies immune system activation and target organ vulnerability to generate the clinical manifestations of lupus.

The genetic contribution to SLE can be supported by assessing the concordance rate of SLE among mono and dizygotic twins. Monozygotic twins have 30% concordance of SLE whereas the concordance rate is only 3% for dizygotic twins (10 times higher rate of concordance)[1.] The identification of single nucleotide polymorphism in diagnosis of SLE has been markedly elevated in recent years. Two major collaborative genome wide association

studies, one organized by SLE genetics (SLEGEN) and other by Genentech, identified nine new genes associated with SLE[2,3] . Both the above studies have arrived at a common conclusion that, lupus associated genes will encode a specific protein necessary for the proper functioning of immune system. Defective gene leads to the synthesis of a defective protein which in turn is the etiology behind malfunctioning of the immune system. The present study also goes with the conclusion arrived by the previous studies, but introduces a different genetic loci, 2q33.2 of CTLA-4 gene in the disease pathogenesis. The product of this gene is called CTLA-4 protein which is involved in the down-regulation of T-cell activity. The study briefly discusses the effect of CTLA-4 gene polymorphism over the level of encoded CTLA-4 protein and its possible role in clinical manifestation of .the disease.

The initiation of T cell activation requires two separate signals, a primary signal presented by MHC class of molecules and a secondary non-specific signal generated by the co-stimulatory molecules[4,5]. The secondary co-stimulatory signal is the result of interaction between CD-28 molecules on T cells with the B7 family of molecules, B7-1 (CD-80) and B7-2 (CD-86) on the surface of antigen-presenting cells (APCs) [6,7]. A molecule that is a structural homolog of CD-28, cytotoxic T lymphocyte associated antigen-4 (CTLA 4) is also expressed on the surface of T cells. In contrast to CD-28, CTLA-4 has a negative regulatory role on T cells[8,9]. Both CD-28 and CTLA-4 molecules will bind to same ligands on the surface of antigen presenting cells, CD-80 and CD-

86. But the affinity of CTLA-4 for CD-80 and CD-86 is 20 to 50 times higher than that of CD-28.

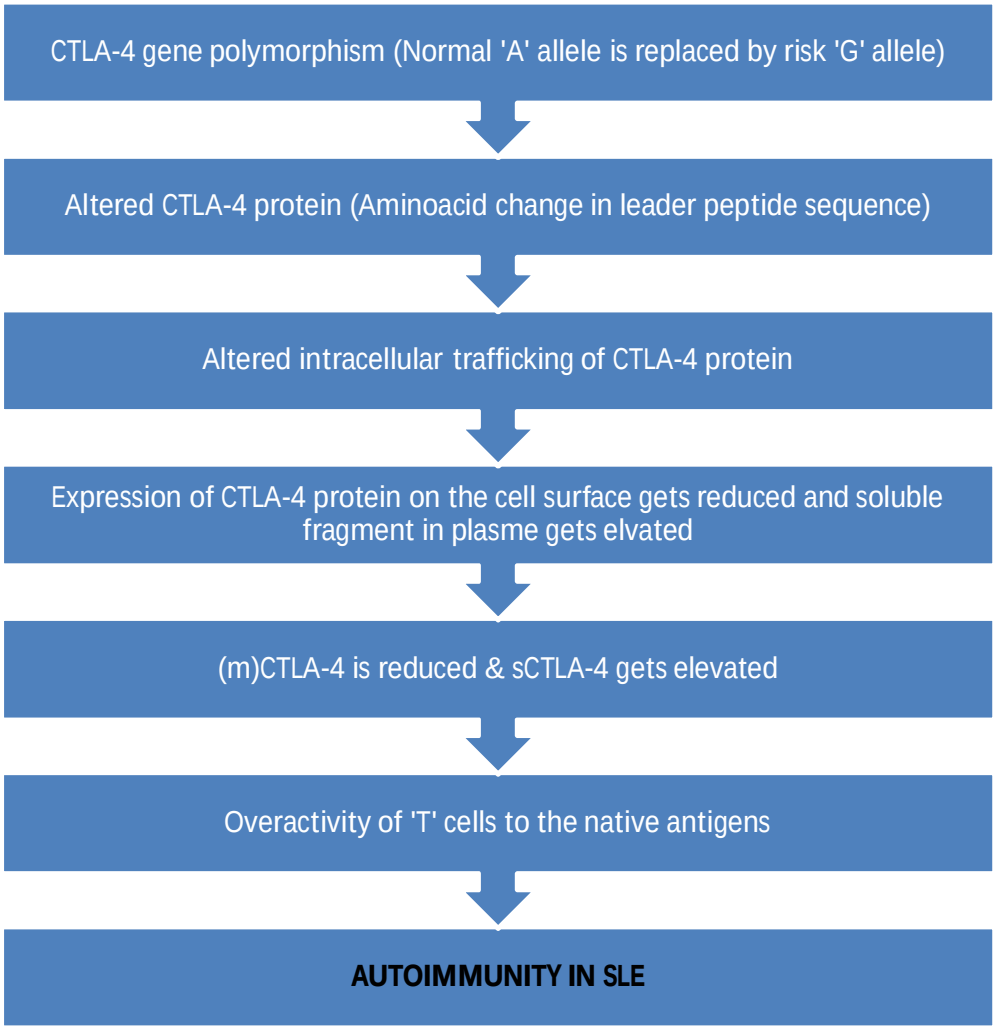
CD-28/B7 co-stimulation is the up regulator for T cell responses whereas CTLA-4/B7 is the down regulator for the development and maintenance of T cells responses[10]. Thus CTLA4 acts as the second receptor for B7[11]. This CTLA-4 mediated negative regulation on T cells is necessary for the induction of antigen specific tolerance[12]. Elimination of this tolerance by specific pathological conditions is responsible for the development of various autoimmune diseases.

The CTLA4 gene of non activated T cells when subjected to PCR produces two different amplicons of size 650 and 550 bp[13]. This is due to the alternate splicing of CTLA-4 mRNA, which will generate a soluble form of CTLA-4 molecule called sCTLA-4. Normal human will have low levels of sCTLA-4. The patients with SLE have increased sCTLA-4 expression on the surface of T cells[14]. sCTLA-4 molecule has the role in pathogenesis of this autoimmune condition[15]. This soluble fragment of CTLA-4 molecule in low levels is responsible for inhibiting the activation of T cells. This is accomplished by blocking the interaction between CD-28 and CD-80/86. On the other hand the same soluble CTLA 4 molecule on higher level will inhibit the binding of membrane CTLA 4 with CD-80/86. This in turn will reduce the inhibitory signal to the T cells. Due to this reduced inhibitory effect there will

be failed inhibition of autoreactive T cell that is responsible for the pathogenesis of SLE.

This case-control study is sought to determine the frequency of CTLA-4 exon 1 polymorphism (+49A/G) among SLE patients and also to correlate it with sCTLA-4 protein levels and disease pathogenesis.

FUNCTIONAL RELEVANCE OF GENE POLYMORPHISM AND THE DISEASE EXPRESSION



Review of Literature

REVIEW OF LITERATURE

Systemic Lupus erythematosus is the prototypic systemic auto-immune disease characterized by diverse multisystem involvement and the production of array of antibodies. Clinical manifestations of the disease is quite variable among individual patients, ranging from mild joint and skin involvement to severe life threatening internal organ damage[16]

EPIDEMIOLOGY:

The Prevalence of SLE has a wide variation among various population groups and sub groups ranging from 20 to 240 per 100,000 persons[17]. This wide variation in prevalence is due to the fact that both geographical location and racial features has significant effect on SLE prevalence. There is comparatively higher prevalence of SLE among Asians, Afro-Americans, and Hispanic Americans compared with Americans of European descent in the US. Due to improved detection of the earlier stage of the disease, many cases of SLE that have earlier gone undetected have now come to limelight and adds to the disease load. So due to this improved rate of detection the incidence rate of SLE has increased four-fold in last 60 years[18]. Estimated incidence rates are in average of 1 to 10 per 100,000 person-years[17,19]. The prevalence is also higher among Asian Indians compared with Caucasians in Great Britain. In European countries compared to Indo-Asians, Caucasians are having 2-3 times lower prevalence rate for SLE[21]. There is 2-3 fold higher prevalence of SLE among Asians/Hispanics compared with white populations[20].

SLE predominantly affects the female population and majority of females are in the reproductive age group. The female to male ratio varies with the age, the ratio being 8:1 in children, 10 to 15:1 in adults and 3:1 in elderly population [21]. Nearly three fourth of the individuals affected with SLE are in the age group of 15-64 years[23]. SLE can also affect the pediatric age group (<15 years of age) and most of the affected children are Africo-American[22]. SLE in pediatric age group and also in male population will be having a more severe progression. The true geographic, racial, and temporal differences in SLE incidence and prevalence may yield important clues to the etiology of disease. [23]

ETIOPATHOGENESIS:

Multiple etiological factors cross-link that leads to the development of disease. It is very difficult to define a specific etiological agent for the disease. Apart from genetic/epigenetic factors that plays a crucial role in the development of the disease multiple ethnic, geographical, immune regulatory, hormonal and many other environmental factors are also responsible for the manifestation of the disease [24].SLE patients exhibit an abnormality in estrogen metabolism, there is increased 16α hydroxylation of estrone in SLE patients to form 16α hydroxyoestrone[25]. Nurses' Health Study which has been done in a large cohort of nurses has proved that, there is increased risk of SLE associated with the use of oral contraceptive pills and Hormone replacement therapy[26,27]. Exogenous administration of estrogen will exacerbate the existing lupus.[28, 29] The major factor responsible for the pathogenesis of SLE is, the self- reactive T

cells, which interact with B cells and promotes B cell differentiation and production of antibodies in SLE. Pathogenic autoantibodies to DNA are frequently associated with autoantibodies to chromatin-associated proteins such as histones or the. The serum examination in lupus patients has shown antibodies complexed to DNA or DNA binding proteins like histones and Ku (p70/p80) antigens.[30]. Although Lupus pathology does depend on immune complex formation and deposition, subsequent investigations identified virtually all cellular components and even many soluble immune system products, that acts as contributors to immune system dysfunction in the disease [16]. One such soluble immune system product to be responsible for pathogenesis of SLE is soluble fragment of CTLA-4 (sCTLA-4) protein whose production is increased due to alternate splicing of CTLA-4 mRNA. Various other deficiencies in T cell function such as defective production of typical T cell cytokine interleukin-2 or defective IL-2 receptor have also been described to play a role in autoimmunity in SLE [31].

CONTRIBUTORS OF SYSTEMIC LUPUS ERYTHEMATOSUS PATHOGENESIS:

The genetic profile of an individual is a major factor in determining the susceptibility to the development of disease. Many disease associated genetic variants are identified and these variants are either associated with increased production of stimulatory nucleic acids or their impaired clearance. Products of innate immune response like type I interferons are also produced in increased amounts. There is an altered threshold of activation or efficiency of signaling

of cells of the adaptive immune response. Multiple genetic variants along with the environmental triggers establish a state of immune activation that leads to the development of autoimmune disease. But in rare cases a single mutation in genes that are the critical regulators of immune system activation are sufficient to the development of altered immune status and the development of autoimmunity.

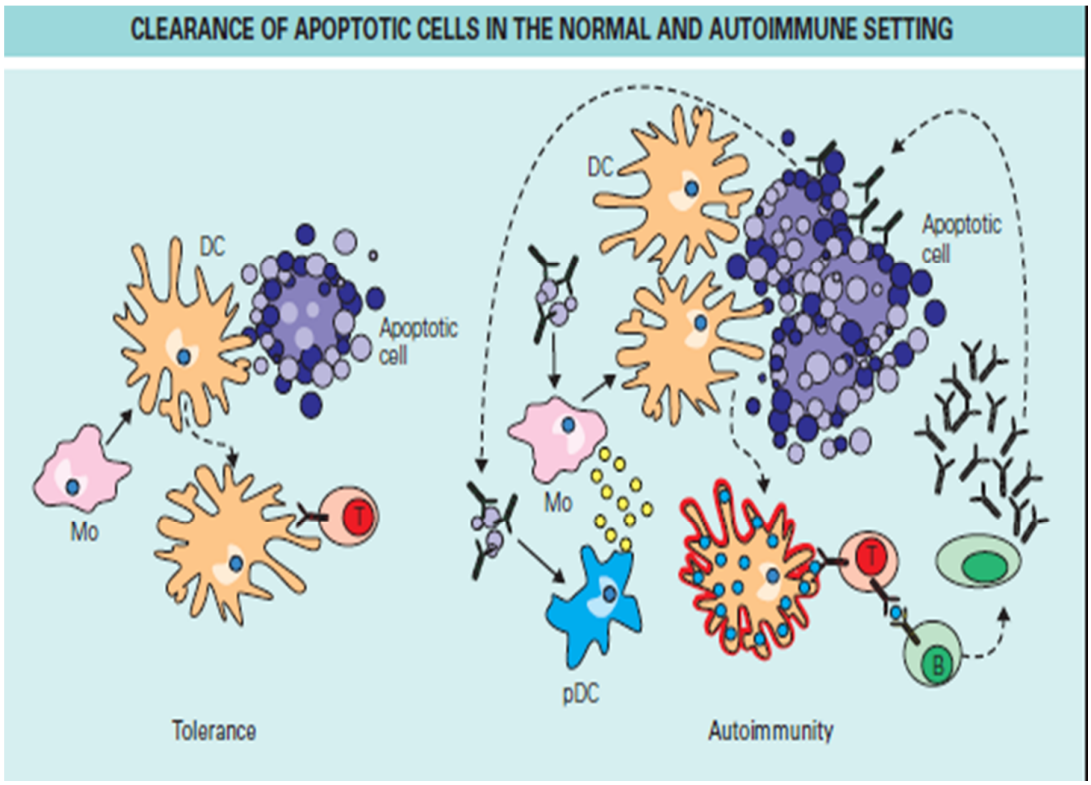
Plasmacytoid dendritic cells (pDCs) are responsible for the production of Type 1 interferons. These cells are activated by intracellular nucleic acids or by exogenous triggers like virus or some other debris. The activation of pDCs might be the contributing factor responsible for the initiation and progression of disease. The production of IFN-α has a numerous effects on the cells of immune system, there will be augmented antigen presenting capacity of myeloid dendritic cells and this in turn will activate self reactive T cells and this leads to the differentiation of B cells and production of pathogenic antibodies. The increased expression of CD-154 on the surface of activated T cells associated with increased release of interleukin-21 will act as stimulus for B cells to differentiate into antibody producing plasma cells. There is also increased production of B cell activation factor (BAFF), which is needed for survival and differentiation of B cells [29]. There is increased autoantibody production from B cells and increased immune complex deposition in the vicinity of the blood vessels. This deposited immune complex will promote the complement activation, inflammation and tissue damage and also IFN-α will

stimulate endothelial cells which is associated with poor vascular repair and sclerosis.

In spite of these above gains in elucidating the pathogenesis of SLE, the environmental triggers and genetic susceptibility factors that lead to the initiation of autoimmunity remain largely undefined in majority of the SLE patients. The major environmental trigger being identified to be responsible for pathogenesis of SLE is ultraviolet light mediated DNA damage and modification of DNA methylation that renders self-nucleic acid stimulatory to the immune system.

In healthy individuals, dendritic cells and macrophages are involved in the presentation of antigens to T cells. In the process auto-reactive T cells will be inactivated. Fc receptors of low affinity, complement receptors of low density, and genetic or acquired deficiency of complement factors diminish the capacity of the immune system of patients with lupus to scavenge immune complexes and apoptotic cell debris. These factors, along with an increased load of apoptotic cells, overload the immune system with auto-antigens. The increased load in a susceptible individual leads to the development of autoantibodies. These autoantibodies opsonize apoptotic cells, which favors an inflammatory manifestation. Additionally, they form immune complexes that stimulate antigen-presenting cells, thus creating a vicious circle (FIGURE 1).

FIGURE 1


CLEARANCE OF APOPTOTIC CELLS IN THE NORMAL AND AUTOIMMUNE SETTING
DC
Apoptotic cell
Mo
T
DC
Apoptotic cell
Mo
pDC
T
B
Tolerance
Autoimmunity

I. GENETIC CONTRIBUTORS TO LUPUS PATHOGENESIS

There are many evidences that document the importance of genetic factors, contributing to SLE. For example, there is a strong familial clustering of the disease, with 10% to 12% of SLE patients having affected first-degree relatives [32]. Data also suggests that there is 10 times higher concordance of clinical lupus among monozygotic twins compared to dizygotic twins, although the highest concordance rate is still only 57% [16].

Genetic variants associated with systemic lupus erythematosus:

- Homozygous deficiency of early complement components (C2, C4A, C4B)- 5 to 10 fold increased risk [33]
- Major Histocompatibility complex genes Associated with SLE: [16]
  - HLA DR 2/ HLA DR 3 – 2 to 5 fold increased risk[34]
  - HLA DR2/DRX - Associated with anti-Sm antibodies
  - HLA DR3/DRX - Associated with anti-Ro and anti-La antibodies
  - HLADR2/DR3 - Associated with anti-Ro, anti-La, anti-Sm and anti-ds DNA Ab
  - HLADR3/DR3 - Associated with anti-Sm antibodies
- Non- MHC genes associated with SLE[16]
  - Homozygous deficiency of C1q
- Association based on linkage studies[16]
  - Fc gamma receptor IIa (FCG2A) [34]
  - Fc gamma receptor IIIa (FCG3A)

- Programmed cell death 1 (PDCD1)

- Association based on candidate gene studies [16]
  - C- reactive protein (CRP)
  - Interferon regulatory factor 5 (IRF 5) [36]
  - Interleukin- 10 (IL- 10)
  - Protein tyrosine phosphatase 22 (PTPN22)

- Association confirmed by genome wide association studies [16]
  - B Cell scaffold protein with ankyrin repeats - BANK 1 gene[37]
  - B Lymphocyte specific tyrosine kinase - BLK gene[38]
  - Tumor necrosis factor- induced protein 3 - TNFAIP3
  - TNFAIP3 interacting protein 1 - TNIP1
  - Signal transducer and activator of transcription factor 4 – STAT 4
  - 3-prime repair exonuclease 1 - TREX 1
  - Integrin Am - ITGAM

SLE associated genes are involved in the generation of self-antigens, innate immune system activation and also activation of adaptive immune system. The deficiency of the genes responsible for the synthesis of complement components like C2, C4 and C1q can also lead to lupus pathogenesis. When this complement components are defective there will be defective clearance of cellular debris, in other words there will be increased accumulation of nuclear debris. This increased nuclear debris will act as self-antigens and stimulate self- reactive T cells that further leads to autoimmunity.

Although GWAS has identified a significant statistical association between variations in genes sequences with diagnosis of SLE, the functional consequence of these variations has not yet been studied. The gene that has been best studied to understand the impact of lupus associated variants on immune cell function are the genes involved in the production of Type I IFN. The risk alleles IRF5 and IRF7 are associated with increased serum type I IFN activity in those patients who demonstrate autoantibodies targeting DNA or RNA associated proteins [39, 40.]

II. FEMALE PREDOMINANCE OF SYSTEMIC LUPUS ERYTHEMATOSUS:

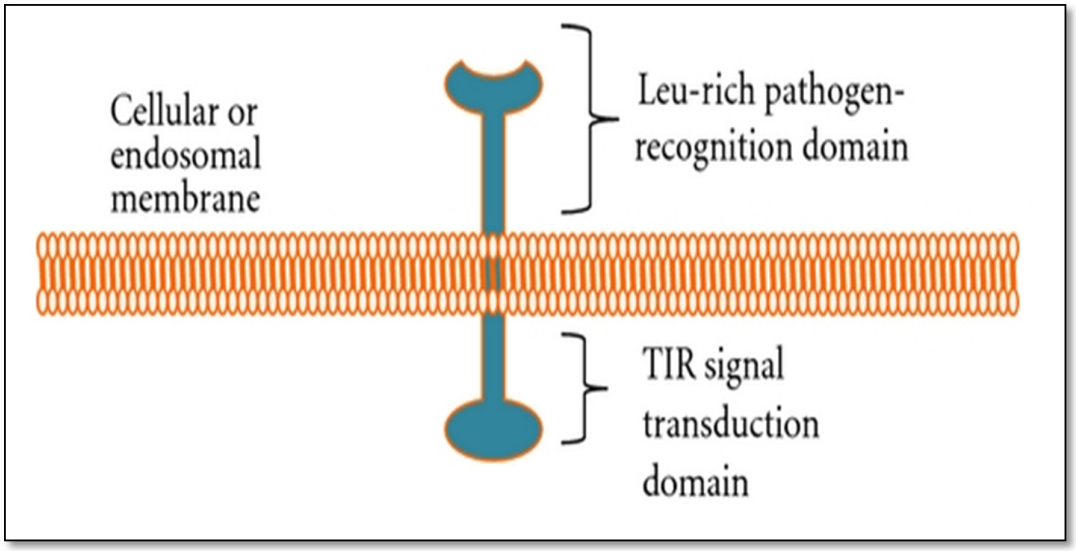
SLE has a dramatic female preponderance in the ratio of 9:1[41]. Hormonal factors play a crucial role in immune system activation which is responsible for the female preponderance of the disease; Elevated levels of estrogen and prolactin will promote the survival and activation of autoreactive B cells[42.] Estrogens will modulate the activation of lymphocytes and prolactin has also been shown expressed at increased levels in serum of lupus patients. There is a positive relationship between early menarche and SLE, and breastfeeding mothers have lower risk of SLE[43]. Men with Klienfelters syndrome, characterized by a 47XXXY genotype has 14 fold increased risk of SLE compared to men without SLE [44]. These data suggest that the dose effect of X chromosome gene is a possible risk factor for development of SLE. The disease has the typical occurrence during the child-bearing age group, after

menarche and before menopause. Also there exists a positive correlation between early menarche and SLE. The above observations that, increased risk of SLE among women who had early menarche and who had not breast fed had led to the hypothesis that factors related to ovulation can contribute to lupus pathogenesis.

III. ENVIRONMENTAL TRIGGERS OF LUPUS:

There is a higher level concordance of SLE among monozygotic twins that ranges from 25-50%[45], which provides evidence to the fact that environmental factors and stochastic events contribute to the development of SLE in an individual. There are two well known environmental triggers that contribute to the pathogenesis of SLE, that are UV radiations and certain drugs. Ultraviolet rays act on the skin cells, breaks the DNA that ultimately leads to altered gene expression. The end result is the apoptotic or necrotic cell death. The impaired clearance of these apoptotic cells is one of the factors leading to the development of UVB induced skin lesions in lupus patients [46]. The likely mechanism for the development of drug induced lupus is that there will be altered DNA methylation[47]. Socioeconomic status plays a role in progression of SLE, patients of low socioeconomic status have poor access to health care hence more severe will be the disease progression[48]. There are also many epidemiological studies that have shown the higher prevalence of EBV antigen specific antibodies in children with SLE. A study among military cohort has supported the fact that higher level of EBV viral load is associated with greater disease pathogenesis[49].

FIGURE 2: STRUCTURE OF TOLL LIKE RECEPTORS



IV. INNATE IMMUNE SYSTEM ACTIVATION IN SLE:

The discovery of TLRs and elucidation of the central role of innate immune system in the regulation of acquired immune system has opened new insights into the disease pathogenesis. TLRs are classified as type I transmembrane proteins that are characterized by an extracellular domain, a transmembrane domain and a cytoplasmic tail. (FIGURE 2)

The extracellular domain will be containing leucine rich repeats (LRRs). The cytoplasmic domain will be containing Toll/IL-1 receptor (TIR) domain, which initiate a signaling cascade to promote immune response[50]

The direct pathogenic role of nucleic acid complexed to immunoglobulins in lupus are proved by studies based on

- nucleic acid sensitive TLRs and
- nucleic acid independent TLR-s

that are responsible for the production of Type I interferons and other proinflammatory mediators. In the peripheral blood cell of the lupus patients, there will be broad expression of type I IFN inducible genes and is referred to as "IFN signature"[51,52,53]. These studies points towards innate immune system as significant factor contributing to lupus pathogenesis.

In summary, the important mechanism for lupus pathogenesis is the combination of increased generation of modified nuclear material with associated impaired clearance of this nuclear material. Recent attention is

focused towards microparticles, that are very small membrane-enclosed particles released by activated and dying cells, has generated a data demonstrating binding of these antigens by several autoantibodies in SLE patients[54]. The deficiency of complement components C1q, C2 and C4 is also strongly associated with SLE. This is because C1q and other complement components are necessary for the clearance of apoptotic debris in SLE[55]. Many newer diagnostic as well as therapeutic modalities based on regulating the complement activity have already been postulated for SLE[56].

V. ADAPTIVE IMMUNE SYSTEM ALTERATIONS IN SLE:

CD4 +T cells produce helper signal to B cells that drive the B cell differentiation and production of autoantibodies. Hence, CD4 + T cells are absolutely essential in the pathogenesis of lupus. Many systematic studies done on T cells of SLE patients have concluded that defect or possible alterations in T cell signaling pathways, release of various cytokines, cellular proliferation, and defect in regulatory functions have possible roles in SLE[57]. Studies of lupus T cells have also given an interesting observation of global epigenetic alterations of lupus genome which results in autoimmunity. On treating mouse and human T cells with azacytidine there is resultant increased expression of the lymphocyte function antigen-1 (LFA1; CD11a) adhesion molecule and associated enhanced proliferative responses to self-non–T cells[58]. Ligation of T cell receptors and T cell activation results in increased intracellular calcium levels, hyperpolarization of mitochondria in T cells[59]. Individuals of HLA 8.1 haplotype have characteristic low IL-2 production[60]. BAFF is survival factor

for B cells and is a TNF family member. BAFF expressed by many different cell types binds to BAFF receptors that are expressed on the surface of cells during development. Many in vitro experiments have been conducted that supported the possible role of cytokines such as IL-21 and B lymphocyte stimulator (BLys) / B cell activating factor (BAFF) and TLR ligands that activate the B cells to produce antibodies ,Out of all the stimulators for B cells, T cells are proved to be the most efficient drivers for B cells [61]. The presence of CD8+T cells with memory phenotype has worse prognosis, due to tissue damage caused by these cells [62]. There is hypo-methylation in the CG- rich areas of the genome, and this has been proved by a recent study on identical twins. The twins were discordant over manifestation of lupus and the twin with active lupus has demonstrated hypo-methylation of the CG rich regions of the genome [63]. Lupus patient have reduced expression of Treg cells and enhanced Th17 cells[64].Treg cells suppress immune responses whereas Th17 cells are responsible for activation of inflammation by activating the production of IL-17.There are many mechanisms that have been postulated to establish the role of DNA de-methylation in T cells of lupus patients. In lupus patients there is increased expression of the protein growth arrest and DNA damage induced 45 alpha (GAD 45 alpha). This GAD 45 alpha protein is responsible for the removal of methyl groups from DNA[47]. There can be decreased ERK pathway signaling due to decreased expression of the protein DNAMT1 protein that leads to DNA de-methylation. There are recent documented evidences that T cells of lupus patients are characterized by the increased synthesis of ICOS,

CXCR5, Bcl6 and also IL-21 that mediate important signal which promotes the differentiation of B cells directed against self antigens[65,66]. PTPN22 acts as adapter molecule in B cells of healthy individuals. Studies on risk variant PTPN22 in lupus patients shows that there is increased selection of autoantibody producing B cells [67]. Apart from impaired regulation of T cells in SLE patients, impaired regulation of B cells can also lead to development of SLE. There is a recent report that proves an association between Vitamin D deficiency and the presence of antinuclear antibodies. The activity of both B cells and type I interferons are elevated in SLE patients with concomitant vitamin D deficiency[68]. The products of the stromal cells and chemokines are responsible for the production of long lived plasma cells. These long lived plasma cells act as the sources for anti-Sm and anti-Ro antibodies in SLE patients. These patients are also refractory to B cell depletion therapy [69]. Plasmablasts in the circulation are the primary sources for anti-dsDNA antibodies, so anti-B cell therapy proves to be effective among these SLE patients [70].

AUTOIMMUNITY IN SLE:

Autoantibodies are the mediators of pathology in SLE due to the formation immune complexes with the native antigens. Antinuclear antibody can be demonstrated in almost all the lupus patients. The antibodies that are more specific for SLE are anti-Smith antibody and anti-ds DNA antibodies. A peptide that contains only the amino acids 83-119 of the SMD1 protein binds to double stranded DNA. This complex will be recognized by T cells and in turn

leads to the production of anti-ds DNA antibodies [71,72]. The antibodies that are not specific for SLE like Anti- Ro, anti- La and anti- SNP antibodies are also characteristically seen in the serum of all SLE patients. Autoantibodies can be detected in the serum of the patients even before the appearance of clinical manifestations. The average time period between the appearance of antibodies and the clinical manifestations of the disease varies between five to six years, and the specificity of the antigen being targeted will expand as the duration of the disease increases[73]. Anti-C1q antibodies are also specific for SLE, which recognize neo-epitopes of C1q that are bound to apoptotic cells[74]. Each antibody in SLE is responsible for a specific manifestation of the disease. For instance the anti-Ro antibodies that are derived from maternal circulation in SLE affected mothers are responsible for lupus syndrome in neonates.

AUTO-ANTIBODIES RESPONSIBLE FOR VARIOUS CLINICAL MANIFESTATIONS IN SLE:

- Nephritis : Anti-ds DNA, Anti-Ro, Anti-C1q, Ids16/6, 31 and GN2
- Vasculitis : Anti-Ro
- Dermatitis : Anti-Ro, Anti-dsDNA
- CNS : Anti-ribosomal P, Anti-neuronal, Anti- NR2
- Lymphopenia : Anti-lymphocyte
- Hemolysis : Anti-erythrocyte
- Thrombocytopenia : Anti-platelet
- Clotting : Anti-phospholipid

- Fetal loss : Anti-phospholipid
- Neonatal lupus : Anti-Ro
- Mild disease : Anti- RNP without other autoantibody except antinuclear antibody

MECHANISM OF TARGET ORGAN DAMAGE:

The target organ damage in SLE is due to activation of complements and also release of products from phagocytes caused due to deposited immune complexes. A recent study has extensively analyzed the renal infiltrating cells at various points of the disease. The study has shown that uncontrolled tissue repair and organ dysfunction in SLE is due to the differentiation of monocytes into a functional phenotype[75]. IFN-α is a contributor to the development of crescents in lupus nephritis [76].

The target organ damaged can be vascular system. The common vascular lesions include peri-arteriolar onion skinning typically seen in spleen, micro-angiopathy in several target organs and also dysfunction of endothelial cells that are responsible for premature atherosclerosis. It has been postulated that increased type I interferons are responsible for impaired vascular repair in lupus patients [77]. The target organ damage in SLE can also been due to granulocytes and pro-inflammatory lipids [78].

CLINICAL FEATURES OF SLE:

Systemic lupus erythematosus is the disease associated with a wide variety of clinical manifestations. The degree of manifestation of each and

every symptom also varies. Some patients will have milder disease while in others the disease rapidly progress to life threatening illness.

Criteria for the classification of SLE was proposed by American college of Rheumatology (ACR) in 1971, this criteria was revised in 1982 and again revised for the second time in 1997 [79,80].

TABLE 1: Criteria for SLE:

SYMPTOM	CRITERIA
1.Malar Rash	Fixed erythema over the malar eminences that can be flat or raised, but typically spare the nasolabial folds
2. Discoid rash	Raised patches that are erythematous, and keratotic scales are adherent over the patches associated with follicular plugging; Older lesion form atrophic scars
3. Photosensitivity	Skin rashes pointing to unusual reaction to sunlight, based on the history given by the patient or observation by physician
4. Oral ulcers	Painless oral or nasopharyngeal ulcer being noticed by physician
5. Arthritis	Non-erosive arthritis typically involves two or more peripheral joints, and there will be associated tenderness, swelling or effusion of joints
6. Serositis	Pleuritis- history of pleuritic chest pain or rub heard by physician or evidence of effusion in pleura OR Pericarditis- As documented by an ECG or rub or evidence of effusion in pericardium
7. Renal disorder	Proteinuria being persistently above 0.5 g/day or above 3+ if exact quantitation is not performed OR Presence of cellular casts

8. Neurologic disorder	Seizures—There should not be any offending drugs or any metabolic derangements like ketoacidosis or uremia or any electrolyte imbalance OR Psychosis-- There should not be any offending drugs or any metabolic derangements like ketoacidosis or uremia or any electrolyte imbalance
9. Hematologic disorder	Hemolytic anemia, associated with reticulocytosis OR Leukopenia-Count being less than 4,000/mm^3 on greater than 2 occasions OR Lymphopenia OR Thrombocytopenia- Platelet count being less than 100,000/mm^3 and there should not be any offending drugs
10. Immunologic disorder	Anti-DNA antibodies **OR** Anti-Sm antibodies **OR** Positive findings for anti-phospholipid antibodies based on: 1. IgG or IgM anti-cardiolipin antibodies are present in abnormal levels in serum 2. Positive lupus anticoagulant, **OR** 3. False positive TPI test or FTA-ABS for at least six months
11. Positive antinuclear antibody	Abnormal titer of the antibody as detected by immunofluorescence or equivalent assay at any point in time but there should not be any offending drugs

A person must fulfill at least 4 out of the 11 criteria to be classified as SLE. The American college of Rheumatology criterion was being developed as a means of classifying SLE patients for the purpose of inclusion in epidemiological as well as clinical studies. A concerted effort was made

recently to further revise the classification criteria, for example, to make lupus nephritis a “stand-alone” criterion and/or add a low complement criterion [16].

Although SLE has variable clinical manifestations the most common presenting manifestations were constitutional symptoms like fever, fatigue and weight loss, cutaneous and articular manifestations.

TABLE 2:FREQUENCIES OF VARIOUS MANIFESTATINS IN SLE [16]

MANIFESTATON	FREQUENCY
Constitutional symptoms (fever, fatigue, weight loss)	90% - 95%
Mucocutaneous involvement (malar rash, alopecia, mucosal ulcers, discoid lesions etc.)	80%-90%
Musculoskeletal involvement (arthritis/athralgia, avascular necrosis, myositis etc.)	80%-90%
Serositis(pleuritis, pericarditis, peritonitis)	50%-70%
Glomerulonephritis	40%- 60%
Neuropsychiatric involvement	40%- 60%
Autoimmune cytopenia	20%-30%

VARIOUS MANIFESTATIONS OF SLE:

Mucocutaneous Involvement:

Mucocutaneous involvement is very common in SLE. Mucocutaneous involvement can be classified as

I. Acute cutaneous lupus erythematosus (ACLE)

II. Subacute cutaneous lupus erythematosus (SCLE)

III. Chronic cutaneous lupus erythematosus (CCLE)

Patients with SLE typically display more than one type of cutaneous manifestations. The hallmark feature of ACLE is butterfly rash in the malar region. The butterfly rash is characteristically macular or papular which is seen symmetrically over the cheeks or the bridge of nose but there is typical sparing of the nasolabial fold. SCLE has characteristic non-scarring photosensitive lesions that can be papulosquamous or annular polycyclic lesions. There is a typical predilection for back, neck, shoulders and extensor surfaces for SCLE. The annular subtype of SCLE is strongly associated with anti-SSA/Ro antibody [81].CCLE is characterized by a variety of photosensitive lesions that ultimately leads to skin atrophy and scarring. The most common sub-type of CCLE is discoid lupus which can be a localized or generalized discoid lupus. Discoid lesions involving the scalp affects mainly the parietal areas and vertex leading to scarring alopecia. Photosensitivity is typical of almost all SLE patients. Photo provocation will cause abnormal skin reactions to UVA, UVB and visible light in greater than 90% of lupus patients [82].

MUSCULOSKELETAL INVOLVEMENT:

Arthritis and arthralgia are the other common clinical manifestations of SLE present in up to 90% of the patients during the course of the disease [83]. Lupus arthritis can involve any joint but characteristically symmetric inflammatory arthritis predominantly involves knees, wrists and small joints of the hand. Hand deformities occurring as a result of arthritis is called “Jaccoud’s like arthropathy” that can even occur over foot. There is also rare co-existence of tophaceous gout in SLE[,84,85,86].

RENAL INVOLVEMENT:

Renal involvement is very common in SLE being a significant cause of morbidity and mortality. Although more than 90% of the patients present with evidence of renal pathology on biopsy only 50% will develop clinically diagnostic nephritis and is a cause of significant mortality and morbidity[87]. Laboratory Evaluation for renal pathology:

Urine analysis

Urine analysis by microscopy is an essential screening test for monitoring of lupus nephritis patients [88]. Proteinuria, Hematuria, dysmorphic RBCs, red blood cell casts and WBC casts are the features seen in routine urine analysis of nephritis patients . Accurate measurement of proteinuria is critical because proteinuria is a very critical indicator of glomerular damage. Studies indicate that in chronic kidney disease patients, strong predictor GFR decline is the magnitude of proteinuria [89]. Normal daily protein excretion is less than 150 mg. The gold standard procedure is to collect 24 hours urine to assess proteinuria. Spot protein to creatinine ratio can be also be used to assess proteinuria. Spot PCR, although not so accurate as 24 hours timed sample, a spot ratio can be a helpful screening test for the detection of proteinuria and is useful in differentiating nephrotic from non-nephrotic range proteinuria[90]. The gold standard procedure for proteinuria assessment is PCR in 12 -24 hour urine[91].

While following up the patients with lupus nephritis, assessment of detoriation in renal function over time is more important than absolute value of renal function tests. Although easy to measure, serum creatinine is an insensitive indicator and trend of serum creatinine over time is the reasonable method to follow the renal function in an individual with lupus nephritis. It is preferable to use Estimated GFR calculated by Cockraft- Gault formula or Modification of diet in renal disease (MDRD) formula. In a SLE patient with clinical or laboratory features suggestive of nephritis, a renal biopsy is an absolute necessary to confirm the diagnosis and also to assess the degree of disease activity.

The degree of SLE glomerulonephritis is classified by the International society of nephrology/ Renal pathology society (ISN/RPS). There are six categories of SLE glomerulonephritis based on light microscopy, immunofluorescence and electron microscopy findings [92].

Class I : Minimal mesangial lupus nephritis

Class II : Mesangial proliferative nephritis

Class III : Focal lupus nephritis

Class IV : Diffuse lupus nephritis

Class V : Membranous lupus nephritis

Class VI : Advanced sclerotic lupus nephritis

PLEUROPULMONARY INVOLVEMENT:

Pleuritis is seen in nearly 50% of the SLE patients. High levels of serum C-reactive protein (CRP) have been found to correlate well with the presence of pleuritis and other forms of serositis in SLE provided no other infections are present in the patient [93,94]. Thus serum CRP provides useful clue for the identification of pleuritis in SLE patients. Shrinking lung syndrome occurs in small set of lupus patients and should be considered while evaluating a SLE patient with unexplained dyspnea and associated pleuritic type of chest pain [95].

CARDIOVASCULAR INVOLVEMENT:

The typical cardiovascular manifestations in SLE include pericarditis with or without effusion, myocarditis, valvular abnormalities and also coronary artery disease. The most common cardiac manifestation seen in SLE is pericarditis. Women with SLE have 50 fold increased risk of myocardial infarction compared to healthy individuals [96].

NEUROPSYCHIATRIC INVOLVEMENT:

Neuropsychiatric lupus (NPSLE) is a broader term that envisages a wide range of neurologic and psychiatric manifestations seen in SLE patients. NPSLE can involve any aspect of central or peripheral nervous system. CNS disorders range from diffuse processes such as acute confusional states, psychosis, headache and mood disorders to more focal processes such as myelopathy, seizures and chorea.

GASTROINTESTINAL INVOLVEMENT:

Any part of the gastrointestinal system can be affected in SLE patients. Dysphagia is the predominant feature seen in over 13% of the SLE individuals. Manometric studies in SLE patients have demonstrated abnormalities in esophageal motility [97]. Abdominal pain due to peritonitis, pancreatitis, mesenteric vasculitis, and intestinal pseudo-obstruction has been noted in 40% of SLE patients.

OPHTHALMOLOGIC INVOLVEMENT:

Keratoconjunctivitis sicca is the most common ocular manifestation in SLE [98]. The abnormalities in retina can be detected by ophthalmoscopic examination, and the retinal abnormalities appear to correlate well with nephritis and CNS manifestations in lupus and also with anti-phospholipid antibodies [99.] Episcleritis and scleritis can also occur in SLE affected individuals.

HEMATOLOGIC MANIFESTATIONS:

Hematologic involvement is common in SLE. All the three major blood cell lines will be affected in the disease. Anemia of chronic disease is the most common etiology for anemia in SLE, but anemia can also be due to autoimmune hemolysis or hemolysis due to microangiopathy. Leukopenia occurs in approximately 50% of SLE patients. Mild thrombocytopenia can be noted in up to 50% of the SLE patients due to autoimmune platelet destruction.

Thrombocytopenia in SLE is almost always correlated with antithrombopoietin antibodies[100]

Lymphadenopathy and Splenomegaly are also the common manifestations in SLE. No individual laboratory test or a clinical manifestation is specific for the diagnosis of SLE. Instead, the disease is being diagnosed by the constellation of characteristic symptoms, signs and laboratory findings.

SEROLOGICAL MARKERS IN SLE:

Serological tests are more important in the diagnosis of SLE patients. SLE is characterized by production of wide variety of auto-antibodies that are crucial for the diagnosis of SLE [101]. The presence of antinuclear antibodies is the hallmark serologic feature in the diagnosis of SLE. But positive ANA is also seen in other autoimmune disorders like rheumatoid arthritis and Type I Diabetes mellitus. Elderly individuals exhibit low titers of ANA even in the absence of autoimmune disorders [102] It is necessary to identify the target nuclear antigen in the patients with positive ANAs. The prominent antibody seen is the Anti-dsDNA which is seen in around sixty percentage of the SLE patients. In fact presence of Anti-dsDNA is highly specific for the diagnosis of SLE. Anti-Sm antigen similar to anti-dsDNA is also highly specific for the diagnosis of SLE, but the antibody is present only in 30% of lupus patients. The other antibodies that can be detected in SLE patients include Anti-RNP antibodies, cytoplasmic anti-Ro and anti-La antibodies. Unlike anti-dsDNA and Anti-Sm antibodies, the sensitivity and specificity of cytoplasmic antibodies

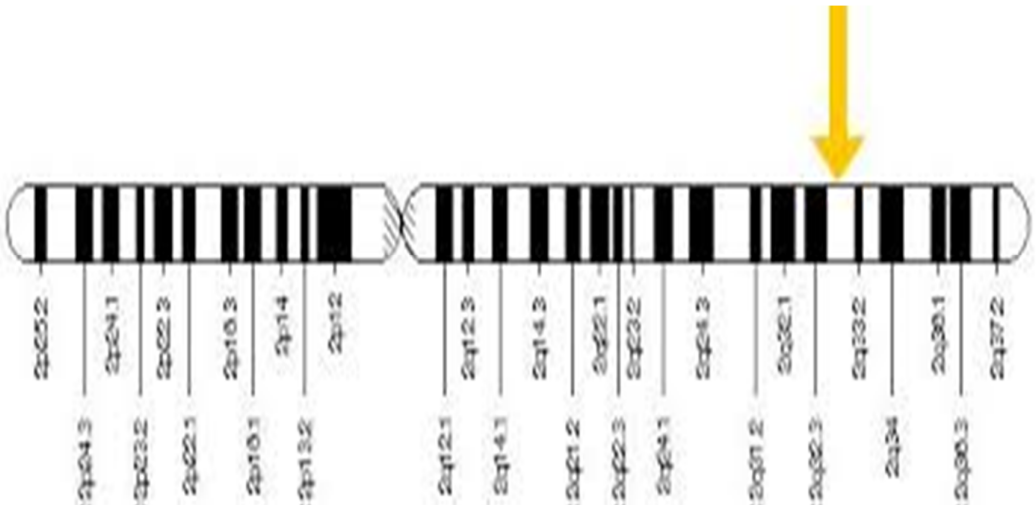
are very low. More than 90% of neonates exhibiting lupus manifestations have associated anti-Ro antibodies[103,104.] 15-20% SLE patients have positive Rheumatoid factor of anti-IgG variety regardless of the presence of arthritis[105]. Anti- CCP antibodies can also be seen in SLE. The patients with active disease have hypocomplementemia, due to complement consumption by the immune complexes[106,107]. SLE affected individuals are also reported to have hereditary complement deficiency of complement components C1q, C2 and C4, so reduced complement components in SLE is not always reflection of the complement consumption.[108, 109, 110] The degree of hypocomplementemia and titers of anti-ds DNA correlates with disease activity in SLE. There are many systemic disorders that mimic SLE. Hence before diagnosis of SLE, a comprehensive search of other autoimmune disorders especially an extensive genetic study is needed to diagnose SLE.

CTLA-4 PROTEIN

Cytotoxic T Lymphocyte associated protein 4 (CD-152), a molecule expressed on the surface of helper T cells is a member of the immunoglobulin superfamily and is a transmitter of inhibitory signal to T cells. The protein was first identified by screening the complementary DNA library of murine T cells[111]

The genes for CD28 and CTLA-4 are having similar structures[112] and the cytogenetic location is 2q33.2 (FIGURE 3)

FIGURE 3: CTLA-4 GENE STRUCTURE AND LOCATION:



The gene for CD28 and the gene for CTLA-4 have also been linked at the molecular level. Genomic studies have proved that they are found on the same artificial chromosome of the yeast bearing human genomic DNA, and these two genes are separated from each other only by a distance of 25-150 kb[113]. There is approximately 20% homology in base sequence between CD28 and CTLA-4 genes but there is striking 31% homology in aminoacid sequence between CTLA-4 and CD28 protein. This homology of base sequence between CTLA-4 and CD28 genes and their close proximal location has led to the hypothesis that, the two genes CTLA-4 and CD28 are the products of duplication event from a single gene.

The CTLA-4 protein has an

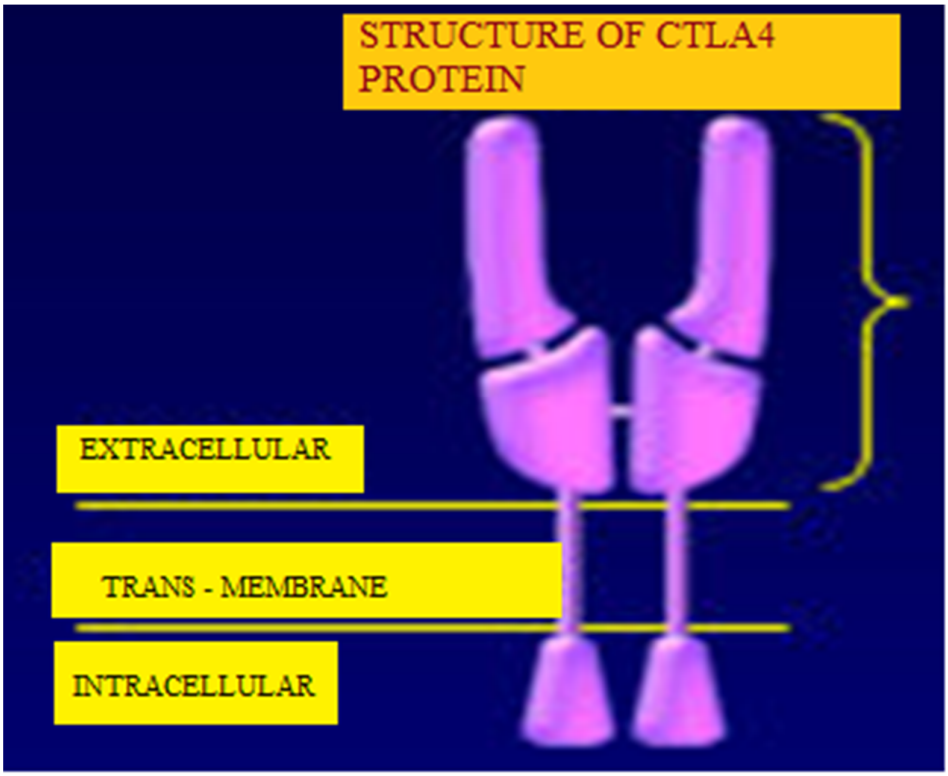
- An extracellular V shaped domain,
- A trans-membrane domain and
- A cytoplasmic domain (FIGURE 4).

The molecular weight of the protein is 24, 656 Daltons. Alternate splicing of CTLA-4 gene will produce a variety of isoforms of CTLA-4 protein.

The two major isoforms of the protein are the

- The membrane bound, and
- The soluble isoform.

FIGURE 4: STRUCTURE OF CTLA-4 PROTEIN



The soluble isoform of CTLA-4 protein is a monomer. The membrane bound isoform of the protein is a homo-dimer and the two monomeric units are interconnected by a di-sulphide bond.[114]

The intracellular domain of CTLA-4 protein is similar to CD28 and has no intrinsic catalytic activity. The domain has two different motifs.

- A YVKM motif and
- A proline rich motif.

The YVKM motif is able to bind PI3K, PP2A and SHP-2 and the proline- rich motif is able to bind SH3 containing proteins.

FUNCTION OF CTLA-4 PROTEIN:

T cell receptors are necessary to identify a wide variety of unknown or known pathogens that can attack us from the universe. But some T cell receptors are even disadvantageous that they recognize our own antigens, so that the entire T cell becomes self-reactive against our own tissues. Thymus offers some degree of protection against the generation of self-reactive T cells. But the protection offered by the thymus gland is necessarily incomplete and a good proportion of self-reactive T cells are able to reach the peripheral circulation. In peripheral circulation CTLA-4 protein plays the key role in controlling self-reactive T cells. This above fact is being supported by an in-vitro study which states that, mice genetically deficient in CTLA-4(CTLA-4 knockout mice) has profound immune dysregulation and autoimmune disease [115]

MECHANISM OF ACTION OF CTLA-4 PROTEIN:

Treg cells the $CD4^+$ T cells are responsible for suppressing potentially deleterious activities of helper T cells, and are the major type of cells expressing CTLA-4 marker on their surface[116]. The CTLA-4 molecule does not function in isolation, instead it requires CD28 molecule, the related glycoprotein for its active function. (FIGURE 5) Engagement of both TCRs and CD28 molecule on the surface of T cells with their respective ligands leads to T cell activation. The T cell activation in turn leads to increased surface expression of CTLA-4 molecule. The expressed CTLA-4 molecule acts as a negative regulator of T cell function[117]

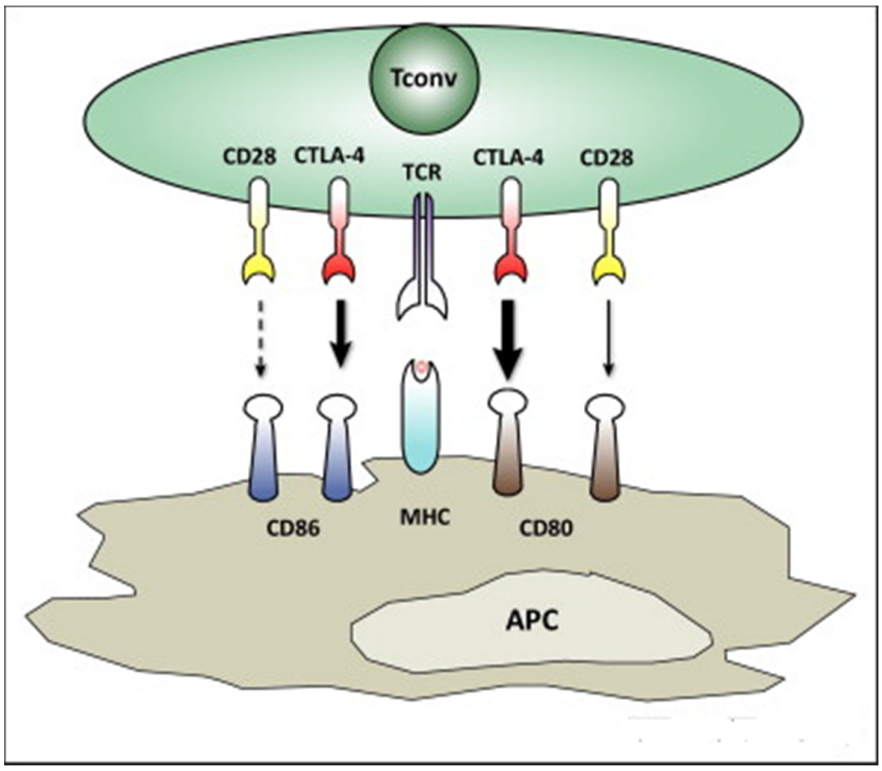
There are a variety of mechanisms being proposed by which CTLA-4 protein cause inhibition of T cell responses

1. CTLA-4 protein binds with greater affinity to B7 family of ligands CD80 and CD86 with much higher affinity compared to T cells, so that CD 28 mediated T cell activation is dampened.
2. The localization of CD28 molecule to the cell surface is disrupted by CTLA-4 protein.
3. A series of phosphorylation events in the intracellular domain of CTLA-4 protein will cause inhibition of T cell function

SHP-2 and PP2A bonded to intracellular domain of CTLA-4 protein will cause de-phosphorylation of proteins such as CD3 and LAT. This CD3 and

FIGURE 5

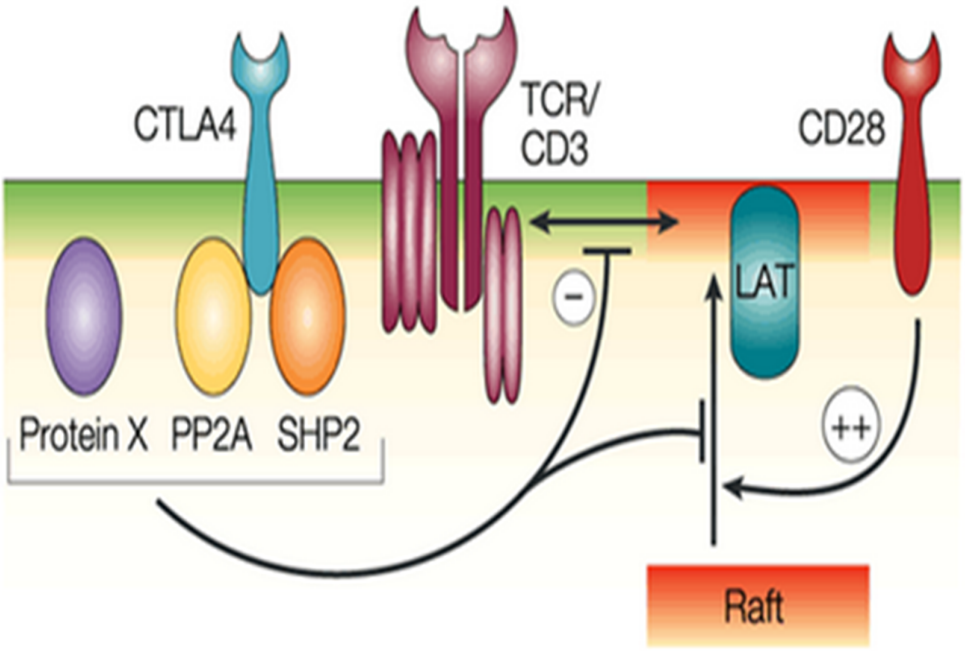
T CELL RECEPTORS AND CO-RECEPTORS ON THE SURFACE OF T CELLS:



LAT proteins are responsible for transmission of stimulatory signal from T cell receptors to interior of the cell. As a result of SHP-2 and PP2A mediated de-phosphorylation of CD3 and LAT proteins, the signal transmission from T cells receptor is blocked leading to the inhibition of T cells (FIGURE 6). In fact a more significant and interacting CTLA-4 partner is serine/threonine phosphatase PP2A[118]. Apart from SHP-2 and PP2A, YVKM motif of intracellular domain of CTLA-4 protein also binds P13K. But the role played by bonding of CTLA-4 protein to P13K in inhibiting T cell responses has not yet been elucidated

CD 28 is the most powerful co-stimulatory molecule discovered so far. When T cell receptors alone are stimulated without concomitant activation of CD 28 there will not be stimulation of T cells. This ultimately may land up in T cell anergy[119]. The signals transmitted from both the T cell receptors and CD28 will integrate at some point inside the T cell and will activate the genes responsible for proliferation of specific T cells. The CTLA-4 protein by directly antagonizing CD-28, controls the excess stimulation of CD-28 molecules in response to antigenic stimulus. So, in case of CTLA-4 deficiency fatal autoimmunity will be observed due to excess stimulation of CD28 molecule by its ligands CD80/86. This auto-immunity triggered by CTLA-4 loss can be prevented by deletion of the ligands CD80/86 or CD28, the co-stimulatory receptor [120,121].

FIGURE 6: MECHANISM OF ACTION OF CTLA-4 PROTEIN:



In case of low level expression of CD80/86 molecules, there will not be any activation of T cells by APCs. There exists a strong competition between CTLA-4 and CD28 molecules for the ligands CD80 and CD86 molecules and the strong inhibitory signals from CTLA-4 molecule will overwhelm the weak stimulatory signal from CD28 molecule[122]

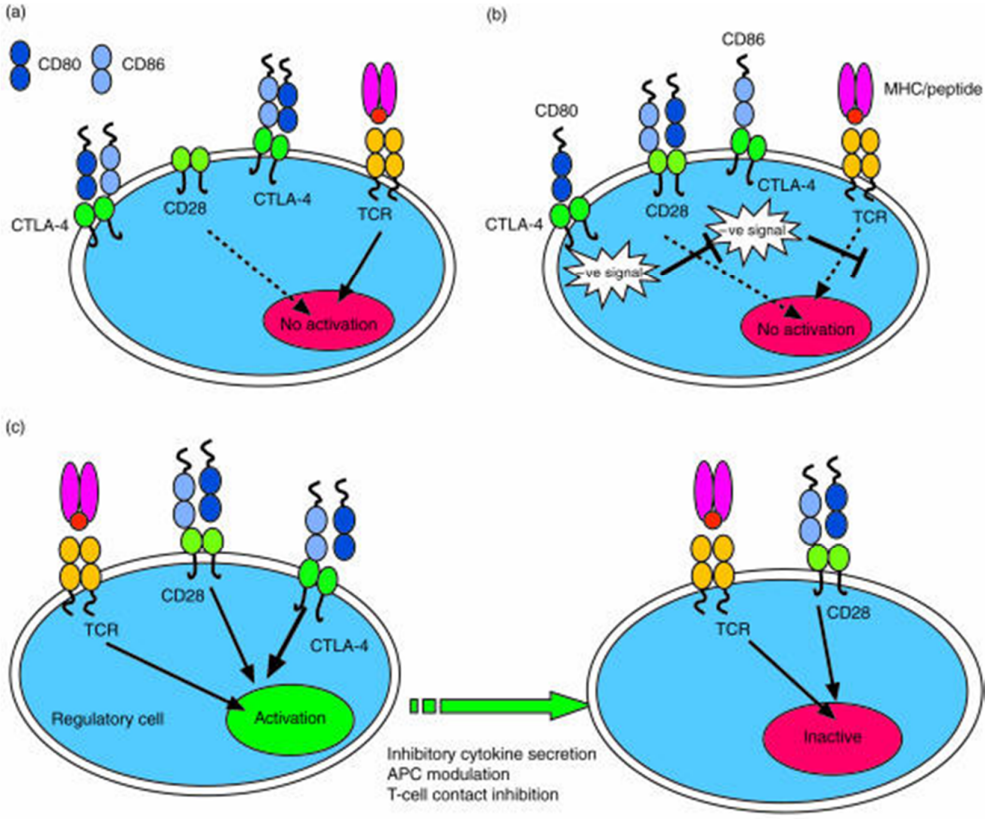
ROLE OF CTLA-4 IN THE REGULATION OF T CELL FUNCTION:

The figure indicates that antigen with lower level of affinity for TCRs cannot activate T cells, because of the strong inhibitory effect from CTLA-4 protein that overwhelms the weak stimulatory effect of the antigen. On the other hand if T cell encounters an antigen with high level of affinity for T cell receptors, then full activation of T cells will follow. This is because sustained positive signaling from T cell receptor and CD28 will overwhelm the negative signaling from CTLA-4. Antigen presenting cells with high level expression of CD80 and CD86 molecules can also cause full blown activation of T cells. The activation of T cells is followed by increased expression of CTLA-4 protein on the surface of T cells. In fact CTLA-4 protein peaks approximately 48-72 hours following activation of T cells, so that now CTLA-4 protein mediated signaling dominates T cell response leading to inhibition of IL-2 production from T cells and so the cell cycle progression comes to a standstill.[122] (FIGURE 7)

ROLE OF CTLA-4 PROTEIN IN AUTOIMMUNITY:

CTLA-4 protein mediated inhibition of T cells is responsible for tolerance developed by T cells against the native antigens. Whenever this

FIGURE 7: CTLA-4 MOLECULE IN THE REGULATION OF T CELL FUNCTION:

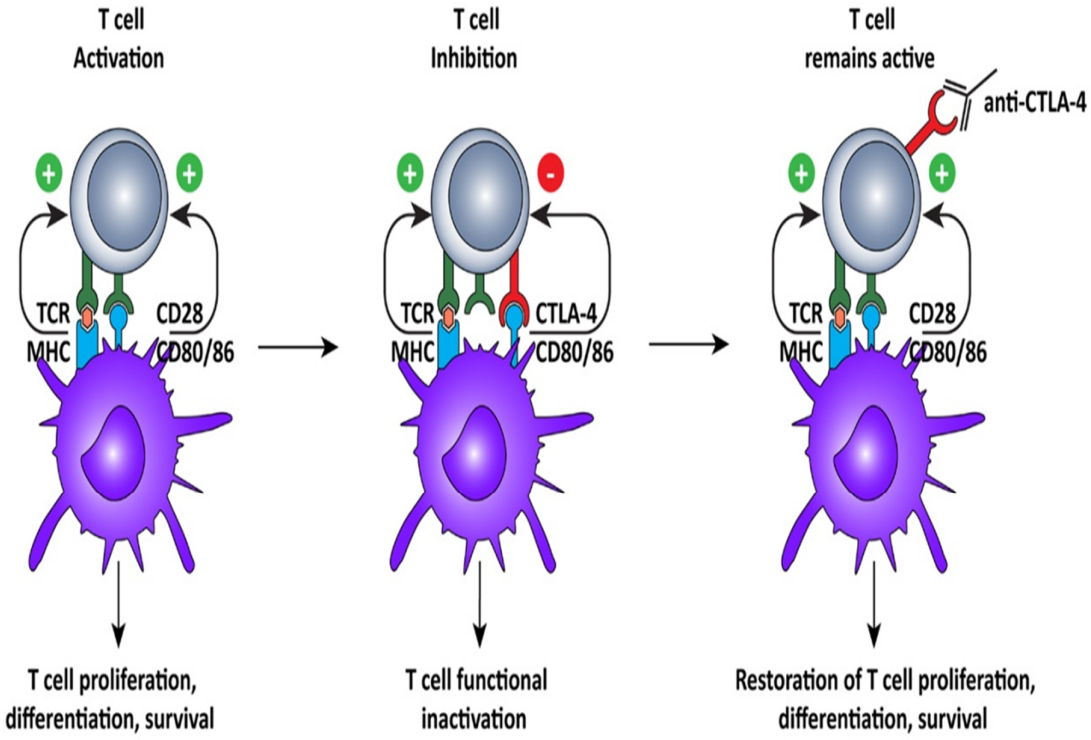


peripheral mechanism of T cell tolerance is eliminated, that is any pathological process that leads to inhibition of CTLA-4 function will end result in proliferation of T cells against self-antigens and lays the foundation for the development of variety of autoimmune disorders[123]. Hence the in-vivo tolerance of T cells to native antigens is due the presence of actively functioning CTLA-4 protein. The signal transduced from CTLA-4 molecule can potentially inhibit signal from TCR or CD28 or both.

CTLA-4Ig:

The CTLA-4 molecule has no effect on signal transduction if the particular cell lacks CD28 co-stimulatory molecule. This can be proved by injection CTLA-4Ig by parenteral route. Anti-CTLA-4 monoclonal antibody (CTLA-4Ig) binds to CTLA-4, so that its interaction with CD28 is blocked, which is now free to interact with CD80/86, prolonging the cellular activation and thus augmenting the immunity mediated by T cells.[124] In normal healthy individuals as indicated in the figure CTLA-4Ig will cause proliferation of T cells by effectively blocking the CTLA-4 mediated inhibitory signals (FIGURE 8). But the Immunoglobulin directed against CTLA-4 protein has no role on T cells if the T cells lack CD28 molecule. The naive T cell requires some degree of TCR stimulation, for CTLA-4 to mediate its inhibitory effect. The absence of TCR stimulation is incompatible with life.

FIGURE 8: FUNCTION OF CTLA-4Ig



CELL SURFACE EXPRESSION OF CTLA-4 MOLECULE:

CTLA-4 molecule is believed to have restricted expression on the surface of CD4 and CD8 T cells, but there is a recent study reporting the expression of CTLA-4 molecule on the surface of B cells and Thymocytes [125,126]. It has been proved that similar to CD8, CTLA-4 molecule is a homodimer linked by disulphide linkages. But many recent reports have proved that CTLA-4 molecule can also exist as a monomer[127.] Resting T cells will not have any surface expression of CTLA-4 molecule. The activation of T cells by antigen presenting cells result in up-regulation of CTLA-4 molecule on the surface of T cells[128, 129]. On encountering an antigenic stimulus there occurs the T cell activation which is then followed by surface expression of CTLA-4 molecule which peaks by approximately 48 hours and returns to normal level by 96 hours[130]. CTLA-4 mRNA expression starts within one hour of T cell activation by antigenic stimulus[131]. A more recent report about CTLA-4 protein is that, it is found localized inside the intracellular vesicles and will cycle between intracellular stores and cell surface depending on the need of the cell[132]. CTLA-4 expression on the cell surface is also mediated by endocytosis by clathrin[133]. The interaction between cytoplasmic domain of CTLA-4 and clathrin associated protein AP-2 is responsible for rapid clearance of CTLA-4 from the cell surface[134]. FACS analysis shows that activated T cells have much higher level of CTLA4 protein than being estimated. The ligands for CTLA-4 molecule are CD80 and CD86, expressed on the APC which is similar to CD28[135, 136,137].

DIFFERENTIAL AFFINITY BETWEEN CTLA-4 AND CD28 FOR LIGANDS

There is a hexapeptide motif, MYPPPY, in the extracellular domain of CTLA-4 molecule, which is also seen in CD28 molecule. This hexapeptide motif is responsible for interaction of CTLA-4/CD28 with the ligands CD80 and CD86[138]. The CTLA-4 molecule has much higher affinity for the ligands compared to CD28. This increased strength of binding is due to the presence of non-conserved residues CDR1-and CDR3-analogous regions in CTLA 4 molecule[138]

THE IN-VITRO EVIDENCES FOR CTLA-4 MOLECULE AS A NEGATIVE REGULATOR OF T CELLS:

Both CTLA-4 and CD28 molecules have extensive homology and are restricted only to the surface of T cells, hence grouped together as co-stimulatory molecules. Many in vitro experiments have improved our current level of understanding of CTLA-4 function and provided strong evidence for the negative regulatory role of CTLA-4 molecule over T cell function

- Artificially cross-linking CTLA-4 molecule with its ligands leads to inhibition of T cell
- Artificially cross-linking CTLA-4 molecule with its antibody leads to enhanced activation of T cell

- Blocking interaction between CTLA-4 molecule and its natural ligands CD80/CD86 with neutralizing antibodies will again lead to enhanced activation of T cells.
- CD80mIg is an immunoglobulin fusion protein in which the extracellular domain of CD80 is found fused to Fc domain of murine Immunoglobulin[139]. When this immunoglobulin (CD80mIg) is added to CTLA-4 molecule, the resultant effect is the blockage of release of IL-3 from CD 4+ effector T cells

MECHANISM OF CTLA-4 SIGNALING:

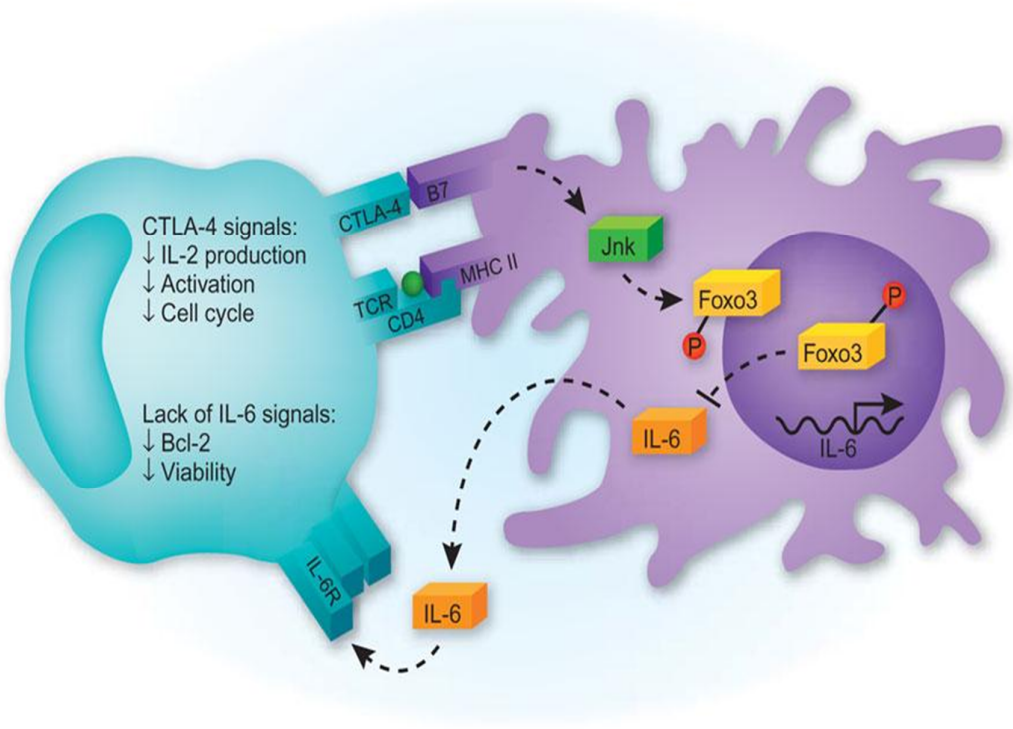
The elucidation of the mechanism by which CTLA 4 molecule mediates its inhibitory effect on T cells is only in the beginning stage.

a) CTLA-4 molecule as a mediator of Apoptosis:

CTLA-4 as a mediator of apoptosis has been proposed by several studies. T cells on activation by specific antigens leads to increased expression of CTLA-4 molecule which in turn leads to apoptosis of activated T cells[140].

Antigen activated T cells will have their CTLA-4 molecule being engaged to B7 molecules on the surface of antigen presenting cells. This results in the generation of series of signals that ultimately down-regulate the release of IL-2 from activated T cells resulting decreased activation of T cells. The engagement of CTLA-4 molecule and B7 molecule generates a series of signals both in the T cells and the Antigen presenting cells.

FIGURE 9: CTLA-4 MOLECULE AS A MEDIATOR OF APOPTOSIS



- Foxo3, a molecule present in the nucleus of antigen presenting cell gets activated as a result of interaction between CTLA4 and B7. This Foxo3 molecule will control the proliferation of T cells by limiting the availability of cytokine IL-6 to T cells, which is essential for the survival of these cells. T cells exposed to limited Interleukin- 6 has lower survival advantage and are more prone to apoptosis.
- T cells are highly dependent on cytokine IL-2 for survival, Interaction between CTLA-4 and B7 results in the inhibition of release of Interleukin -2 from T cells resulting in clonal deletion of T cells. (FIGURE 9)

b) Non-apoptosis mediated cell signaling by CTLA-4:

There are many studies that contrasted the hypothesis that CTLA-4 molecule mediates apoptosis of T cells. Instead the signal from CTLA-4 molecule has few major effects on activated T cells

- Blocks the synthesis and release of Interleukin 2
- Blocks the expression of Interleukin 2 receptor
- Blocks the cell cycle progression of activated T cells [141,142].
- CTLA-4 molecule will recruit a phosphatase to the surface of T cells and this phosphatase will in turn inhibit the signal from T cell receptors.
- Motility of T cells and signals through PI3 kinase can also be modulated by CTLA-4,that leads to inhibition of T cells
- The SH-2 domain of PI-3 kinase and SH-3 domain binds to the cytoplasmic region of the CTLA-4 molecule triggering the sequence of events that ultimately results in inhibition of T cells.

- A recent work has suggested that CTLA-4 molecule will capture and remove B7-1 and B7-2 from the membrane of antigen presenting cells, making them unavailable to trigger CD-28.

C) Reverse stop signaling model for CTLA-4:

T cell motility through normal lymph nodes was studied through multi-photon microscopy, which gave evidence for reverse stop signaling model[143] .This model proposes that there is reversal of TCR induced stop signal by CTLA4 molecule. The stop signal generated by T cell receptor is necessary for firm contact between TCR and antigen presenting cells (APC). By reversing this stop signal, CTLA-4 molecule mediates reduced interaction between T cells and antigen presenting cells.

Although there were many hypotheses being proposed for the mechanism of action of CTLA-4 molecule, the exact sequence of events from binding of CTLA-4 molecule and its ligands to the inhibition of T cells still remains a maze to be solved. Even before the detection of the surface expression of CTLA-4 molecule by flow cytometry, the inhibitory effects of CTLA-4 molecule were detected on the T cell.

The CTLA-4 mediated termination of T cell is responsible for prevention of activation induced T cell death as the protein inhibits the over activity of T cells in response to antigenic stimuli. CTLA4 molecule by preventing antigen induced cell death is responsible for the generation of antigen specific memory cells [144].

ROLE OF CTLA-4 ANTIBODY:

Soluble intact anti-CTLA-4 monoclonal antibody or its F (ab) fragment alone has the same role when injected. Both CTLA-4 monoclonal antibody and its F(ab) fragment will inhibit the CTLA-4 molecule from interacting with its natural ligands CD-80/86. The first study that was conducted to analyze the effect anti- CTLA-4 mAb in human has reported that, antigen induced expression of T cells will be enhanced by both intact CTLA-4 mAb and its Fab fragment [145]. CTLA-4 antibody activates both CD4+ T cells as well as CD8+ T cells.

The neutralizing antibody can be used therapeutically to enhance the action of T cells:

- There will be will be enhanced expression of CD8+ T cells in response to the stimulation by super-antigen of staphylococcus, staphylococcal enterotoxin B (SEB) in the presence of CTLA-4 monoclonal antibody [146].
- CTLA-4 blockade by monoclonal antibodies in combination with melanoma-specific vaccines has been investigated as a potential means of treating advanced melanoma[147]. Ipilimumab and tremelimumab are the CTLA-4 monoclonal antibodies with promising antitumor activity in patients with advanced melanoma. These monoclonal antibodies acts as 'immune checkpoint' which interrupts signal mediated by CTLA-4 and thus inhibits the inhibitory signal to T cells.[148]

There are many recent studies which in contrast to older literatures have reported that, CTLA-4 protein could inhibit the activation of T cells even if CD28 molecule is absent[149, 150]

SOLUBLE CTLA4 PROTEIN:

In the year of 2000, Oak and Hallet first described the alternate transcript of the CTLA-4 gene, which lacks the trans-membrane region of the native CTLA-4 protein[151]. This protein is then called by the name soluble CTLA-4 protein (sCTLA-4). Subsequently, there are many studies that identified the increased expression of the protein in many autoimmune disorders like systemic sclerosis, SLE and myasthenia gravis[152, 153]. sCTLA-4 mRNA has been reported to be constitutively expressed on non-stimulated T cells and its expression is being down-regulated after activation of T cells. But the level of sCTLA-4 protein is elevated in the serum of patients with autoimmune disorders. The biological significance of such sCTLA-4 elevation is that, the protein blocks the interaction of CD80/86 with CD28 thus inhibiting the T cell activation in early stages. Also the sCTLA-4 protein in increased levels will block the interaction between membrane CTLA-4 with CD80/86 ligands. This results in reduced inhibitory signals to T cells. This double edged nature of sCTLA-4 is responsible for pathogenesis of autoimmune disorders

SOLUBLE vs. MEMBRANE CTLA4 PROTEIN:

sCTLA-4 plays a more important role than membrane (m)CTLA-4 in the early stage of the immune response. sCTLA-4 is constitutively expressed in

plasma, while (m)CTLA-4 is expressed on the surface of T cells only upon activation the activation of T cell by APCs. Also, sCTLA-4 has been shown to have immune regulatory properties *in vitro.* Increased production and subsequent secretion of CTLA-4 into the plasma may start in the very early phase of the disease. sCTLA-4 may block the interaction between B7 (CD80, CD86) on APCs and mCTLA-4 on T cells, thereby interfering with the inhibitory signal sent to T cells to enhance the immune response. On the other hand, sCTLA-4 may also bind B7 expressed on APCs and thus interfere with B7:CD28-mediated co-stimulation of T-cell responses. sCTLA-4 along with soluble CD28 plays important roles in modulating the activation of T cells, thereby leading to the exacerbation of disease activity

SINGLE NUCLEOTIDE POLYMORPHISM:

Single nucleotide polymorphism is the DNA sequence variation occurring in only 1% of the population. The DNA sequence variation is such that, a single nucleotide differs between the members of a biological species or between the paired chromosomes. For example, a single nucleotide polymorphism can replace nucleotide cytosine(C) with nucleotide guanine (G) within the stretch of DNA.

On an average a single nucleotide polymorphism can occur once every 300 nucleotides. So there is possibility of 10 million single nucleotide polymorphisms in the entire human genome. There are few SNPs that are proved to be associated with the development of disease. SNPs can also predict

individual response to certain drugs and their susceptibility to various toxins. The SNPs are the focus of intense research recent days, because they help to locate the genes that are associated with the development of disease. SNPs occurring within a gene or in the promoter region of the gene plays a more direct role in disease expression by affecting the expression of the gene.

ARMS PCR:

The amplification refractory mutation system, also known as allele specific polymerase chain reaction is a simple as well as rapid methodology for the detection of single nucleotide polymorphisms. This method was originally described by newton et al in 1989[154] ARMS is based on the use of sequence specific PCR primers that allow amplification of DNA only when target allele is present in the sample. So following ARMS PCR the presence or absence of PCR products indicate presence or absence of target allele. [155] In Tetra primer ARMS PCR, also designated as T-ARMS PCR, has two sets of primers

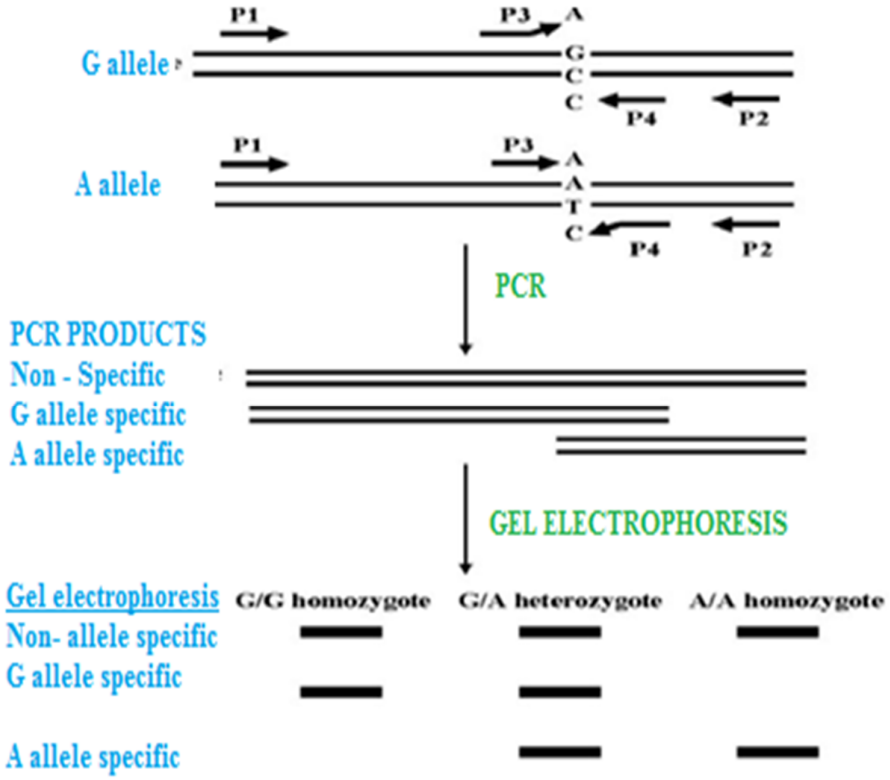
INNER SET OF PRIMERS

- ✓ Allele specific forward primer
- ✓ Allele specific reverse primer

OUTER SET OF PRIMERS

- ✓ Non allele specific forward primer
- ✓ Non allele specific reverse primer

FIGURE 10: TETRA PRIMER ARMS PCR



Diagrammatic representation for the tetra-primer ARMS-PCR method for the detection of single nucleotide polymorphisms

So in T-ARMS PCR, both the wild type and the rare allele are amplified in a single reaction. The outer primer amplifies larger fragment of the target irrespective of the allele present. Inner primer combines with the particular outer primer to generate allele specific amplicons of different sizes. The product can thus be discriminated by gel electrophoresis. (FIGURE 10)

CTLA4 GENE IN SLE:

Many candidate gene studies and genome wide association studies have thrown light on the genetic contributors to SLE. Apart from this, multiple genetic variations play a key role in the development of disease. The common theme proposed by all the gene polymorphism studies in relation to SLE is that, variation in gene sequence leads to the synthesis of a defective protein that acts as a trigger for the development of autoimmunity. CTLA4 is one of the candidate genes whose polymorphism results in defective CTLA4 protein and so disease development. There are four possible polymorphic sites in CTLA4 gene that are reported to be associated with SLE.

- Exon 1 +49 (A/G)
- Promoter -318 (C/T)
- Promoter -1722 (C/T)
- 3'UTR microsatellite (106-bp allele)

Fewer studies have also reported the polymorphism in CT 60 (A/G) gene promoter region can also favor the development of disease. Out of all the above genomic studies exon 1 is so crucial because it is the only coding region

of the gene prone for polymorphism, which codes for the functional CTLA 4 protein. There were extensive research works conducted on this candidate genomic site and reported that the presence of G allele at 49th position of the first coding region (exon 1) of CTLA4 gene is strongly associated with the development of SLE.

Aim of the Study

AIMS AND OBJECTIVES

The aim of the study is

- **To analyze significance of CTLA-4 gene polymorphism (+49A/G) and SLE**

  CTLA-4 gene has 4 exons or coding regions. 49^{th} position in the first exon is prone to polymorphism in which A, the normal allele can be replaced by G, the risk allele, whose significance to SLE is the purpose of this study.

- **To assay the level of soluble fragment CTLA-4 protein and to correlate them with the allelic variation**

  The effect of gene polymorphism on the expression of the corresponding protein is studied. The level of CTLA-4 protein is analyzed among cases and controls and compared between A/A, A/G, G/G genotypes.

Materials & Methods

MATERIALS AND METHODS

This is a case-control study approved by Institutional Ethics Committee (IEC), Madras Medical College and Rajiv Gandhi Government General Hospital.

STUDY POPULATION:

CASES:

Blood sample was collected from 100 proven SLE patients attending Rheumatology OPD (Lupus clinic), Madras Medical College, RGGGH. All the SLE patients selected for the study have satisfied revised ACR criteria for the classification of SLE.

CONTROL:

100 unrelated healthy population who were age and sex matched to the study population have been chosen as controls.

SAMPLE COLLECTION AND SEPERATION:

4 ml of whole blood was being collected in Disodium EDTA tubes. The tubes are then subjected to centrifugation at 3000rpm for 15 minutes. Plasma being separated is used for the estimation of renal parameters. Plasma is also aliquoted and stored at -20 C for the estimation of CTLA-4 protein. After separation of plasma, buffy coat was extracted and transferred into a 2 mL centrifuge tube. From the Buffy coat further DNA extraction was continued on the same day of sample collection.

EXTRACTION OF DNA:

DNA was extracted from separated buffy coat with the help of pure fast human blood genomic DNA Minispin prep Kit

PRINCIPLE

The cells are incubated with proteinase K in the presence of a chaotropic salt. This results in the lysis of the cells and the released nucleases will also be inactivated during this short incubation period. The nucleic acids released from the cells will then bind specifically to the special glass fibres pre-packed in the spin columns. Bound nucleic acids will then be purified by repeated “wash and spin” steps. This will remove any contaminating cellular components. Finally low salt elution will release the nucleic acid from the glass fibre.

PROCEDURE:

All the DNA Extraction steps were carried out at room temperature using a table top micro-centrifuge that can have speed adjusted between 1000-12000 rpm.

Steps in DNA Extraction:

- 20µL of proteinase K is transferred to a fresh 1.5mL centrifuge tube
- 200µL of whole human blood is transferred to the same centrifuge tube and the contents are mixed well by brief vortexing
- 200µL of Lysis buffer is added and the contents of the tube are again mixed well by brief vortexing

- Incubate the micro-centrifuge tube at 56ºC for 15 minutes
- After incubation, 200µL of 100% ethanol is added to each of the centrifuge tubes and the contents are mixed well by vortexing.
- After mixing, a brief centrifugation of 1.5ml centrifuge tubes is done. This will remove drops from inside the lid.
- From the 1.5ml centrifuge tube, the whole sample has to pipetted into the spin column.
- The sample in the spin column is then centrifuged at 8000rpm for 1 minute. The flow through is discarded and the column is again placed back in the same collection tube
- 500µL of ethanol mixed wash buffer-1 is then added to the spin column, and is centrifuged at 8000rpm for 1 minute. The flow through is discarded and the column is again placed in the same collection tube.
- 500µL of ethanol mixed wash buffer-2 is then added to the spin column, and is centrifuged at 12,000rpm for 3 minutes. The flow through is discarded along with collection tube
- The spin column is then transferred to a fresh 1.5mL micro centrifuge tube
- 100µL of elution buffer is then added to the centre of the spin column membrane without touching the membrane with pipette tip
- The contents are incubated at room temperature for 2 minutes and then centrifuged at 8000rpm for 1 minute

- The DNA from spin column is now eluted out into the centrifuge tube. The centrifuge tube can now be discarded
- The DNA being separated is stored at -20º C for future use

IDENTIFICATION OF EXTRACTED DNA:

The DNA being extracted was identified by agarose gel electrophoresis

PROCEDURE:

The agarose to be used for DNA Electrophoresis should be low EEO agarose. The DNA after extraction is subjected to electrophoresis and visualized under UV trans illuminator.

A proper size tank and tray is selected.

- 0.24g of agarose is weighed and is transferred to an Erlenmeyer flask. 30mL of TAE buffer (pH-8.3) is then added to agarose.
- Place the flask in microwave and allow it to boil for 60 seconds
- Allow the contents to cool for few minutes and then 2µL of Ethidium bromide (10mg/mL) is added.
- After mixing the contents well, agarose is now transferred to a 30mL tray with comb in place and the gel is allowed to solidify
- After the gel has been set, the tray is placed inside the electrophoresis tank which is filled with TAE buffer (pH-8.3) (same buffer used for gel preparation)
- Then mix the extracted DNA along with loading dye, Composition- 10mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene

cyanol FF, 60% glycerol, 60mM EDTA , (3µL DNA and 2µL Loading dye), and load the contents into the well.

- Power pack is connected to the tank and the constant voltage of 60V is applied (7V/cm)
- The DNA in the sample, along with 1kb ladder is allowed to run for half an hour.
- The bands are then visualized under UV transilluminator (FIGURE 11)

From the extracted DNA, the amplification of CTLA4 gene was carried out by tetra-primer ARMS PCR methodology.

ARMS PCR:

The major advantage of ARMS PCR over conventional PCR is that, the procedure apart from amplifying the target gene similar to conventional PCR, will also produce different sized products depending on the allelic variation present. Thus ARMS PCR is the one step process, in which there is no need for further restriction digestion of the amplified products

PRIMERS:

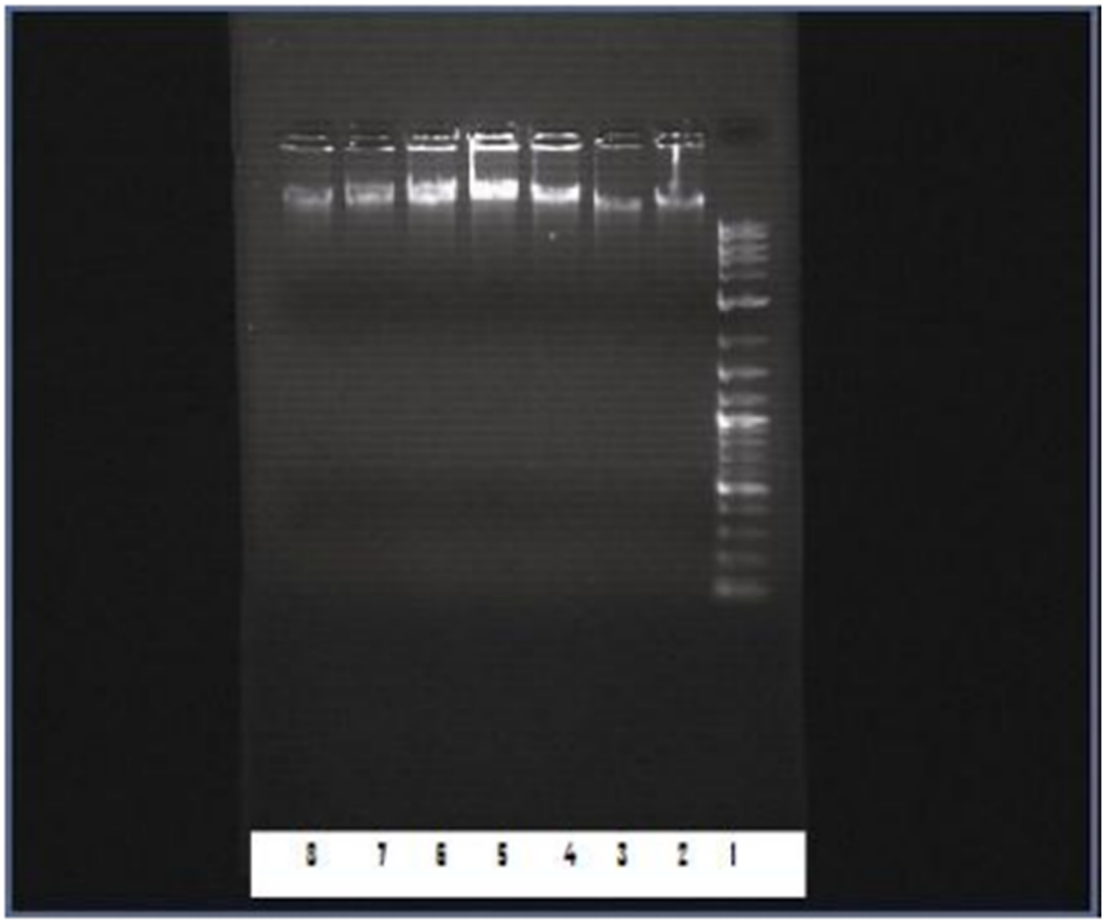
Primer sequence (5' to 3' direction)

- Forward inner primer (G allele-specific)

  5'-AAGGCTCAGCTGAACCTGGATG-3'
- Reverse inner primer(A allele specific)

  5'-AGTGCAGGGCCAGGTCCTTGT-3'
- Forward outer primer

FIGURE 11 : DNA EXTRACTION PRODUCTS



LANE 1: **1 kb DNA LADDER**

LANE 2 to 8: **EXTRACTED DNA**

5'-CCAGCCAAGCCAGATTGGAGTTTTA-3'

- Reverse outer primer

5'-CATCCATGGATTGGCTTGTTTTGTTCA-3'

PROCEDURE:

Before mixing, all the reagents are thawed completely and then briefly centrifuged. The mixing of the reagents is being carried out inside laminar flow hood. TABLE 3:The reagents for ARMS PCR are used in the following composition.

REAGENT	VOLUME
Red dye master mix	10µL
A allele specific primer	2.5 µL
G allele specific primer	2.5 µL
Purified DNA sample	10µL

The total reaction volume is 25µL.

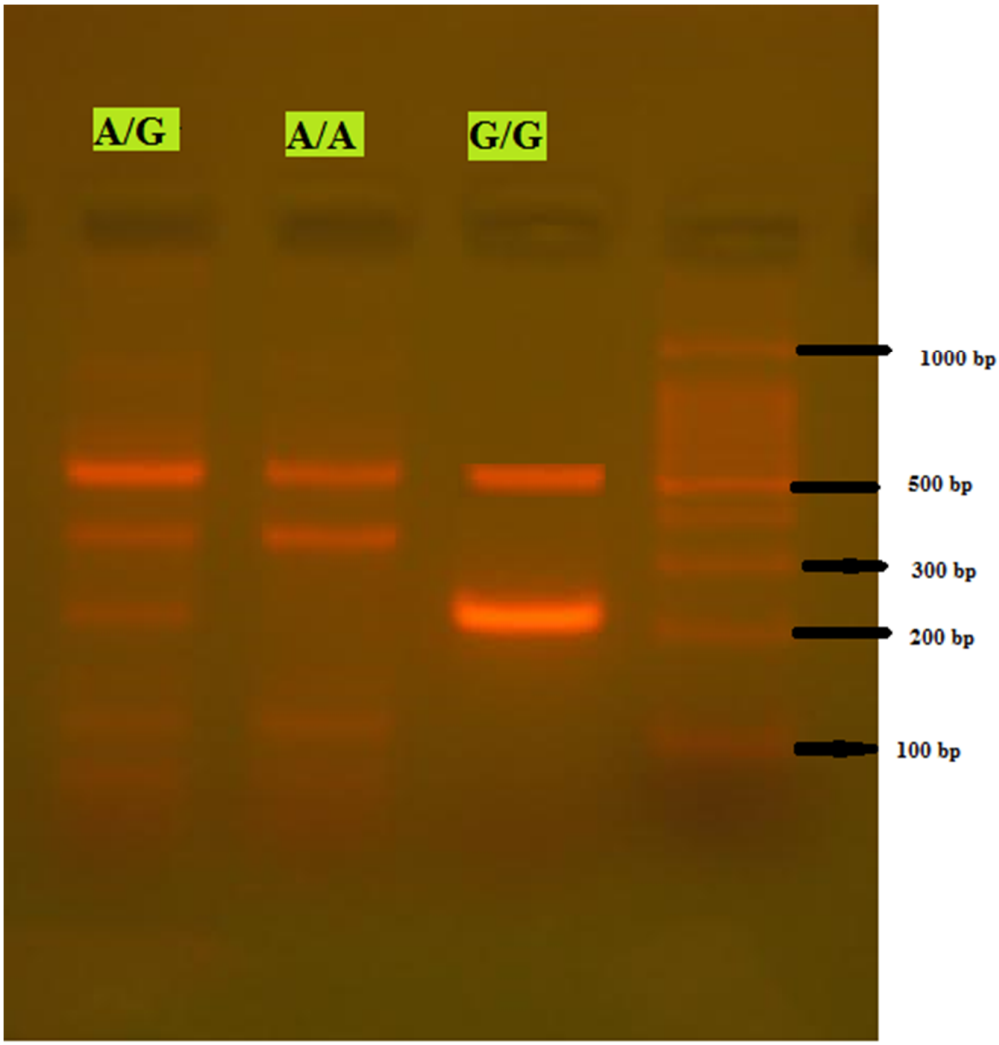
AMPLIFICATION PROTOCOL FOR ARMS PCR:

MyGenie 96 model Bioneer Thermocycler was used to carry out amplification of CTLA4 gene

TABLE 4: CONDITIONS FOR PCR:

	STEP	TIME	TEMP
	INITIAL DENATURATION	5min	95ºC
35 CYCLES	DENATURATION	30sec	95ºC
	ANNEALING	30sec	58ºC
	EXTENSION	30sec	72ºC
	FINAL EXTENSION	5min	72ºC

FIGURE 12: ARMS PCR PRODUCTS



PCR PRODUCTS SIZE:

Homozygous A/A- 350bp & control 575bp

Homozygous G/G- 225bp & control 575bp

Heterozygous A/G- 225bp, 350bp & control 575bp

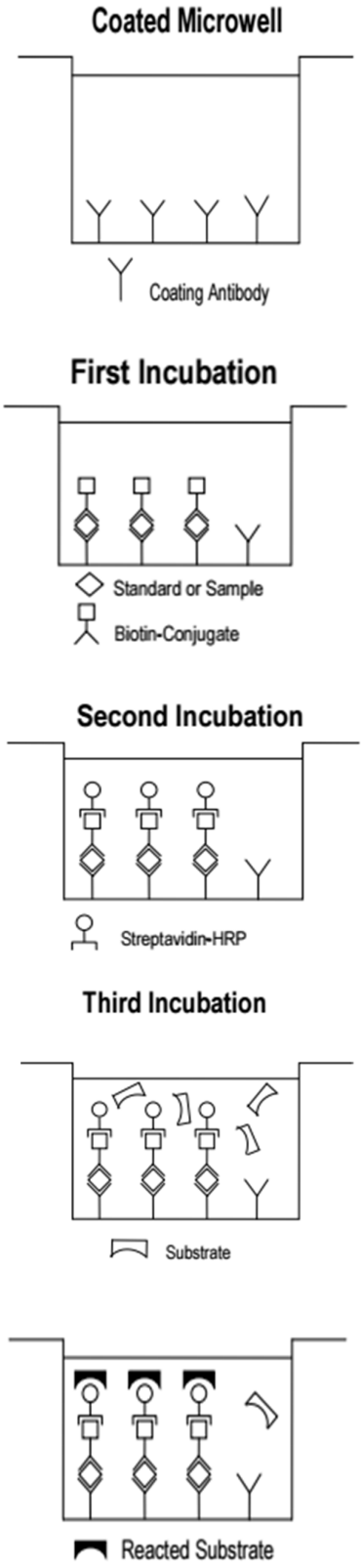
The amplified CTLA4 gene products are then visualized under 2.5% agarose gel electrophoresis. The Electrophoresis protocol was same as explained above except for the concentration of agarose. Entire PCR products are loaded into the well and 10µL of 100bp DNA Ladder is used as control (FIGURE 12).

Estimation of Human sCTLA-4 protein by ELISA:

PRINCIPLE

Microwell plates are coated with anti-human sCTLA4 antibody. The sCTLA-4 protein present in standard or sample binds to antibodies adsorbed to the microwell plates. Another anti-human sCTLA-4 antibody which is biotin-conjugated is then added. This antibody now binds to the human sCTLA-4 captured by the adsorbed antibody. Wash step removes the unbound biotin-conjugated anti-human sCTLA-4 antibody. Streptavidin-HRP then added will binds to biotin which in-turn is conjugated anti-human sCTLA-4 antibody. Again washing will remove unbound Streptavidin-HRP.A substrate solution is added to the wells that reacts with horse radish peroxidase. Reaction between substrate and enzyme results in the production of colored product, whose intensity is proportional to the amount of human sCTLA-4 present in the

FIGURE 13: ELISA PRINCIPLE



sample. The reaction is then terminated by addition of an acid and absorbance of the colored product is measured at the wavelength of 450 nm. A standard curve is prepared from 7 human sCTLA-4 standard dilutions. sCTLA-4 protein concentration in the sample is then determined. (FIGURE 13)

PREPERATION OF STANDARDS:

570µl of distilled water is added to the standard vial provided. The concentration of the reconstituted standard is 20 ng/mL

Take seven test tubes and label: S1, S2, S3, S4, S5, S6, S7. Then 1:2 serial dilutions of standards are prepared for the standard curve

In the test tube labeled S1,

- Pipette 225 µl of Sample Diluent into each tube.
- Pipette 225 µl of reconstituted standard into the first tube and mix

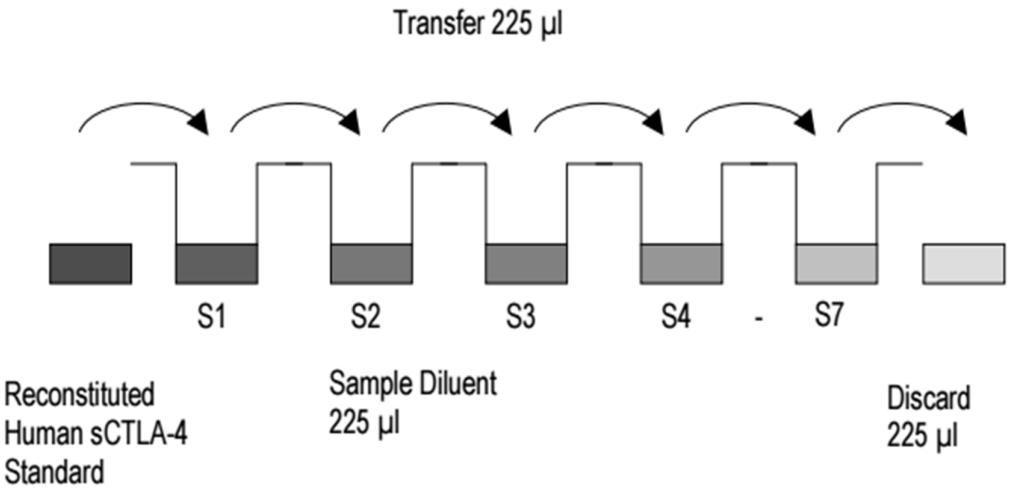
Concentration of standard 1 = 10 ng/ml

- Pipette 225 µl of this dilution into the second tube, labeled S2, which contains 225 µl of sample diluent. Mix well before the next transfer.

Repeat serial dilutions 5 more times thus creating the points of the standard curve

Sample Diluent serves as blank(FIGURE 14)

FIGURE 14:PROTOCOL FOR STANDARD DILUTION



PROCEDURE:

- The microwell plates are washed twice with approximately 400 µl of Wash Buffer. The plates are washed well with thorough aspiration of microwell contents between washes. Wash Buffer is allowed to sit in the wells for about 10 – 15 seconds before aspiration. After the last wash step, wells are emptied and microwell strips are tapped on an absorbent pad so as to remove the excess wash Buffer.
- Pipette 100 µl in duplicates to the standard wells S1 to S7. 100 µl of Sample Diluent is added in duplicate to the blank wells

TABLE 5: CONCENTRATION OF STANDARDS

Sample Well	REPLICATE 1	REPLICATE 2
A	Standard 1 (10ng/mL)	Standard 1(10ng/mL)
B	Standard 2 (5ng/mL)	Standard 2 (5ng/mL)
C	Standard 3 (2.5ng/mL)	Standard 3 (2.5ng/mL)
D	Standard 4 (1.25ng/mL)	Standard 4 (1.25ng/mL)
E	Standard 5 (0.63ng/mL)	Standard 5 (0.63ng/mL)
F	Standard 6 (0.31ng/mL)	Standard 6 (0.31ng/mL)
G	Standard 7 (0.16ng/mL)	Standard 7 (0.16ng/mL)
H	Blank	Blank

- 90 µl of Sample Diluent to the sample wells and add 10 µl of each sample in duplicate to the sample wells. Biotin-Conjugate is then prepared. Add 50 µl of Biotin-Conjugate to all wells.

Cover with an adhesive film and incubate at room temperature (18 to 25℃) for 2 hours

- Streptavidin-HRP conjugate is then prepared
- Remove adhesive film and empty wells. Wash microwell strips 3 times
- Add 100 µl of diluted Streptavidin-HRP to all wells, including the blank wells.

Cover with an adhesive film and incubate at room temperature (18° to 25℃) for 1 hour,

- Remove adhesive film and empty wells. Wash microwell strips 3 times
- Pipette 100 µl of TMB Substrate Solution to all wells.

Incubate the microwell strips at room temperature (18° to 25℃) for about 10 min. Direct exposure to intense light is avoided. The color development on the plate should be monitored and the substrate reaction stopped before positive wells are no longer properly recordable. Alternatively the color development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 – 0.95.

- Stop the enzyme reaction by quickly pipetting 100 µl of Stop Solution into each well.
- The Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read

immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.

- Read absorbance of each microwell on a ELISA reader using 450 nm as the primary wave length and optionally 620 nm as the reference wave length

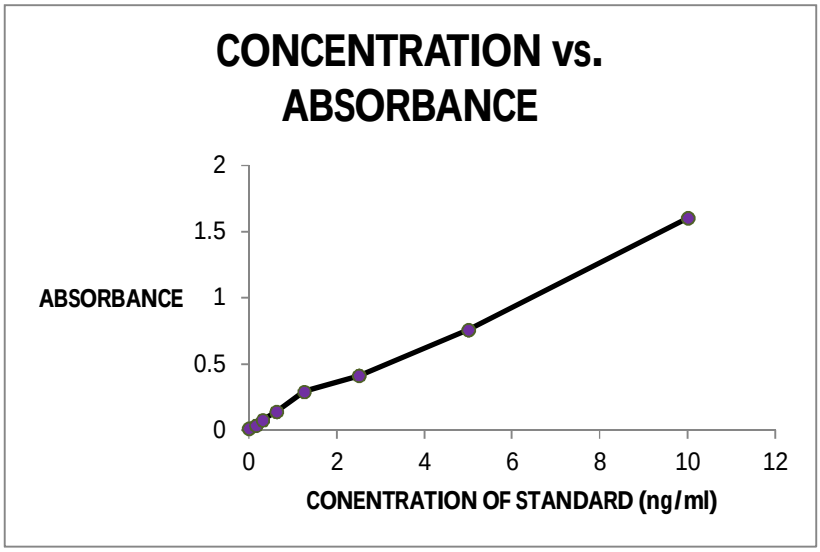
CALCULATION OF RESULTS:

A standard curve is drawn by plotting the mean absorbance for each standard concentration on the ordinate against the human sCTLA-4 concentration on the abscissa. A best fit curve through the points of the graph is drawn. The samples have been diluted 1:10 (10 µl sample + 90 µl Sample Diluent), the concentration read from the instrument is multiplied by the dilution factor (x 10).

TABLE 6: STANDARD CONCENTRATION AND O.D. AT 450 nm:

Standard	Concentration of standard(ng/ml)	O.D. at 450 nm
1	10.00	1. 601
2	5.00	0.759
3	2.50	0.412
4	1.25	0.292
5	0.63	0.142
6	0.31	0.078
7	0.16	0.037
8	0	0.015

STANDARD CURVE:



ANALYTES

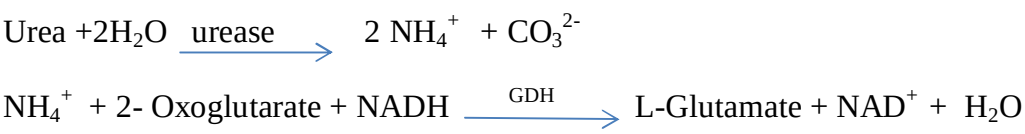
I. UREA:

Mode of assay:

Fixed time Assay

Principle:

Urea is acted upon by the enzyme urease to release two molecules of NH_4^+. The NH_4^+ ions will cause reductive amination of α-ketoglutarate to glutamate in the presence of the enzyme glutamate dehydrogenase. The reaction is coupled to the oxidation of NADH to NAD^+. The NADH but not NAD^+ is able to absorb light of wavelength 340nm. So the oxidation of NADH to NAD^+ is accompanied by decrease in OD at 340nm which is proportional to the concentration of urea in the solution



PROCEDURE:

The assay is done in robonik semi autoanalyzer of model prietest TOUCH. It is the fixed Time assay in which change in absorbance of the sample is measured at the interval of 60 seconds. The initial absorbance is measured after a lag time of 20 seconds and final absorbance after the kinetic interval of 60 seconds. The rate of change in absorbance is measured. The concentration is then calculated from the factor.

II. CREATININE:

Mode of Assay:

Fixed Time assay

Principle:

Jaffe's reaction

Creatinine reacts with picric acid in alkaline medium to give orange yellow color. The absorbance is measured at the wavelength of 505nm which is proportional to the concentration of urea in the sample.

Procedure:

The assay is done in robonik semi autoanalyzer of model prietest TOUCH. It is the fixed Time assay in which change in absorbance of the sample is measured at the interval of 60 seconds. The initial absorbance is

measured after a lag time of 20 seconds and final absorbance after the kinetic interval of 60 seconds. The rate of change in absorbance is measured. Factor has been calculated by using standard. The concentration of creatinine in the sample is then calculated by multiplying the factor with the sample absorbance.

III. URIC ACID

Mode of assay:

End point assay

Principle:

Trinders method:

$$\text{Uric acid} + O_2 + H_2O \xrightarrow{\text{Uricase}} \text{Allantoin} + CO_2 + H_2O_2$$

$$\text{TOOS} + 4\text{AAP} + 2H_2O_2 \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + 4H_2O$$

Procedure:

The assay is done in robonik semi autoanalyzer of model prietest TOUCH. It is the end point assay in which absorbance is measured at the end 5 minutes external incubation. The concentration of uric acid in the sample is assayed by multiplying the factor with the sample absorbance.

Statistical Analysis

STATISTICAL ANALYSIS

Statistical Analysis was done using SPSS software version 20 (p Value<0.05 is stastically significant)

1. Age, Sex, BMI, Serum urea, serum creatinine and uric acid levels were compared between the cases and controls by unpaired student t- test
2. Allele frequency was calculated by allele counting
3. Hardy Weinberg equilibrium was assessed by chi- square test
4. Genotype frequency and allele frequency between cases and controls were compared by chi square test
5. Odds ratio for G allele distribution among the study population is calculated
6. Plasma CTLA-4 protein level between cases and controls was compared between study groups by unpaired student t- test
7. Plasma CTLA-4 protein level for the each of the three CTLA-4 genotype (A/A, A/G, G/G) was compared by one way ANOVA
8. Age of onset of the disease among GG and non-GG genotype individuals were compared by student t-test

Results

MASTER CHART FOR CASES										
S.no	AGE (years)	SEX	DM	HT	BMI	UREA (mg/dL)	CREATININE (mg/dL)	URIC ACID (mg/dL)	CTLA-4 PROTEIN (ng/mL)	CTLA-4 GENOTYPE
1	33	F	A	A	23.38	28	0.9	4.1	77	A/G
2	34	F	A	A	21.67	36	1.1	3.7	88.9	A/G
3	29	F	A	A	24.58	46	1	7.7	120.3	G/G
4	37	F	A	A	29.91	38	0.8	4.5	63.5	A/A
5	26	F	A	A	31.21	45	1.2	7.8	109.7	G/G
6	28	M	A	A	20.07	44	1.5	6.7	111.3	G/G
7	53	F	P	A	23.38	52	1.4	5.5	96	A/G
8	32	F	A	A	29.12	18	1	7.4	127	G/G
9	18	F	A	A	18.34	48	2.1	6.3	142.4	G/G
10	34	M	A	A	22.22	63	1.9	7.7	83.4	A/G
11	46	F	P	P	25.89	47	1.8	6.2	92.9	A/G
12	37	F	A	A	27.31	31	1	4.5	89.9	A/G
13	33	F	A	A	28.72	53	1.1	6.3	89.3	A/G
14	36	M	A	A	25.91	71	1.6	7.8	88.2	A/G
15	46	F	P	A	26.33	22	1.2	4.3	77.1	A/G
16	26	F	A	A	21.87	54	1.5	5.4	106.7	G/G
17	22	F	A	A	32.89	21	1	3.5	128.3	G/G
18	33	F	A	A	20.07	52	1.3	5.3	37.8	A/A
19	34	M	A	A	23.55	46	0.9	6.7	87.2	A/G
20	39	F	A	A	26.32	30	1.3	4.2	80	A/G
21	39	F	A	A	27.18	45	1.4	5.1	77.1	A/G
22	37	F	A	A	20.45	58	1.2	7.1	73.9	A/G
23	52	F	P	A	22.15	48	1.8	5	45.3	A/A
24	44	M	A	A	23.39	26	0.6	4.3	79.8	A/G
25	40	F	A	A	32.19	50	1.6	6.5	87.6	A/G
26	38	F	A	A	25.32	15	0.4	4.2	83.2	A/G
27	33	F	A	A	27.19	46	1	7.3	89.8	A/G
28	39	M	A	A	29.2	25	0.8	5.5	78.3	A/G
29	30	F	A	A	31.19	55	1.1	7.8	151.1	G/G
30	43	F	A	P	19.45	47	1.1	5.7	66.8	A/G
31	31	F	A	A	29.07	52	1.3	7.2	143.4	G/G
32	35	F	A	A	28.33	36	0.9	4.5	93.9	A/G
33	30	M	A	A	20.67	49	1.3	7.6	98.3	G/G
34	37	F	A	A	23.33	47	1.2	5.4	92.2	A/G
35	28	F	A	A	21.98	55	1.1	7.1	136.2	G/G
36	29	F	A	A	25.32	53	0.9	6.5	125	G/G
37	34	F	A	A	30.07	33	0.7	3.4	91.3	A/G
38	33	M	A	A	29.98	57	0.7	7.1	98.3	A/G

39	39	F	A	A	23.93	53	1.2	5.2	51.2	A/A
40	28	F	A	A	21.89	27	0.6	3.7	113.2	G/G
41	38	F	A	A	29.4	52	1.6	6.3	94	A/G
42	40	F	A	A	28.49	45	1.2	7.1	86.6	A/G
43	48	F	P	A	27.19	81	2.2	12.1	36.5	A/A
44	25	F	A	A	25.47	49	1.5	8.3	108.6	G/G
45	36	M	A	A	32.54	37	1.2	7	86	A/G
46	37	F	A	A	27.38	50	0.7	5.3	87.2	A/G
47	33	F	A	A	28.36	53	1.6	4.8	36.5	A/A
48	24	F	A	A	29.12	49	1.8	5.5	119.8	G/G
49	47	F	A	A	24.33	56	1.3	6.1	82.3	A/G
50	40	M	A	A	25.81	21	1	4.3	43.5	A/A
51	31	F	A	A	25.22	30	1.1	3.8	123.5	G/G
52	32	F	A	A	26.81	49	0.6	9.1	119.2	G/G
53	45	F	A	A	23.32	55	1.3	8.4	86.5	A/G
54	28	F	A	A	29.9	51	1.5	6.5	107.8	G/G
55	36	M	A	A	25.22	45	1.7	5.4	73.2	A/G
56	30	F	A	A	32.13	56	1.6	5.5	118.9	G/G
57	39	F	A	A	31.18	22	0.5	3.2	129.3	A/G
58	45	F	A	A	29.81	53	1.5	7.1	105.6	A/G
59	53	F	P	A	26.88	33	0.7	3.3	49.3	A/A
60	38	F	A	A	23.33	45	0.9	7.5	78.6	A/G
61	37	M	A	A	20.8	52	0.9	5.3	69.8	A/G
62	32	F	A	A	21.11	46	1.2	5.1	88.2	A/G
63	35	F	A	A	22.8	73	3.2	9	85.3	A/G
64	41	F	P	A	29.91	54	1.3	8.3	80.6	A/G
65	47	M	A	A	23.39	65	3.5	7.8	89.2	A/G
66	37	F	A	A	28.86	47	1.1	4.5	49.3	A/A
67	42	F	A	A	25.45	18	0.8	7.8	83.6	A/G
68	34	F	A	A	23.32	55	0.9	8.4	84.9	A/G
69	32	F	A	A	29.01	56	1.2	6.5	123.3	G/G
70	45	F	A	A	20.84	54	1.3	3.4	42.3	A/A
71	35	M	A	A	22.66	36	0.7	9.1	86.3	A/G
72	37	F	A	A	23.44	52	0.8	8.2	82.3	A/G
73	41	F	P	A	21.78	51	1.2	11.1	88.9	A/G
74	43	F	P	A	27.89	57	1.7	13.4	36.3	A/A
75	30	F	A	A	23.98	55	1.6	5.6	129.8	G/G
76	32	M	A	A	31.12	25	1.1	3.7	136.6	G/G
77	32	F	A	A	27.63	32	1.4	8.1	149.3	G/G
78	35	F	A	A	18.12	46	1	4.2	92.3	A/G
79	40	F	A	A	29.99	52	1.6	5.3	99.8	A/G
80	38	F	A	A	20.34	48	1.1	6.1	45.3	A/A
81	33	F	A	A	20.81	42	1	7.2	90.8	A/G

82	42	M	A	A	22.33	49	1.5	5.9	83.6	A/G
83	34	F	A	A	28.45	27	0.8	3.8	82.3	A/G
84	29	F	A	A	20.06	55	1.4	7.8	128.3	G/G
85	20	F	A	A	21.82	30	0.9	4.5	106.5	G/G
86	33	M	A	A	23.33	52	0.8	7.5	84.3	A/G
87	19	F	A	A	20.92	57	1.3	5.4	98.3	G/G
88	44	F	P	A	20.84	51	1.3	8.3	83.2	A/G
89	39	F	A	A	20.32	47	1.2	5.9	57.2	A/A
90	27	F	A	A	29.98	62	2.1	15.1	99.3	G/G
91	15	F	A	A	20.1	49	1.3	8.2	101.6	G/G
92	29	F	A	A	19.2	17	0.5	5.1	108.5	G/G
93	31	F	A	A	29.3	54	1.1	6.2	130.2	G/G
94	31	F	A	A	18.16	23	0.8	5.6	103.8	G/G
95	46	F	P	P	23.39	46	0.9	9.2	86.2	A/G
96	37	M	A	A	20.25	45	0.8	8.6	80	A/G
97	39	F	P	A	23.95	35	0.5	3.4	89.2	A/G
98	54	F	P	P	19.82	53	1.3	5.7	39.8	A/A
99	37	F	A	A	29.43	52	1.1	4.8	76.5	A/G
100	33	F	A	A	26.67	51	1	10	92.3	A/G

P- PRESENT A- ABSENT

MASTER CHART FOR CONTROLS

S.NO	AGE (years)	SEX	DM	HT	BMI	UREA (mg/dL)	CREATININE (mg/dL)	URIC ACID (mg/dL)	CTLA-4 PROTEIN (ng/mL)	CTLA-4 GENOTYPE
1	31	F	A	A	28.59	22	0.8	4.4	29.8	A/A
2	16	F	A	A	22.31	16	0.4	3.1	40.6	A/G
3	26	M	A	A	23.5	29	0.7	4.5	33.8	A/A
4	36	F	A	A	26.3	31	1	5.2	63.8	G/G
5	30	F	A	A	31.9	25	0.9	5	22.3	A/A
6	51	F	P	A	25.54	28	1.1	6.2	28.4	A/A
7	39	F	A	A	20.12	30	0.6	2.3	31.8	A/A
8	43	F	P	A	23.33	17	1.1	3.5	54.9	A/G
9	38	M	A	A	33.56	27	0.8	4	40.2	A/A
10	32	F	A	A	31.11	27	1.2	2.6	56.3	A/G
11	34	F	A	A	27.33	18	1	5.3	23.4	A/A
12	37	F	A	A	25.56	22	0.9	2.4	48.3	A/G
13	35	F	A	A	31.56	33	1.1	2.7	52.8	A/G
14	45	F	P	A	34.66	24	0.6	4.2	66.5	A/G
15	37	F	A	A	21.11	35	0.8	6.8	78.5	G/G
16	33	M	A	A	32.25	13	1.2	4.3	43.6	A/G
17	22	F	A	A	27.11	21	1	3.7	39.8	A/A
18	34	F	A	A	21.34	32	1.1	4.7	28.6	A/A
19	33	F	A	A	23.98	45	1.4	5.3	73.4	G/G
20	39	F	A	A	30.09	30	0.8	4.1	51.6	A/G
21	33	F	A	A	25.57	22	0.9	3.7	53.4	A/G
22	47	F	P	A	32.12	19	0.6	3.6	30.3	A/A
23	37	M	A	A	23.88	21	1	5.1	52.8	A/G
24	39	F	A	A	22.13	37	1.4	6.3	28.3	A/A
25	36	F	A	A	20.45	43	1.5	7.1	30.6	A/A
26	32	F	A	A	23.62	26	1	3.3	45.8	A/G
27	45	F	P	A	28.78	25	0.9	4.1	43.9	A/G
28	40	M	A	A	26.55	17	0.9	3	26.5	A/A
29	37	M	A	A	25.47	30	0.5	2.2	28.3	A/A
30	35	F	A	A	20.01	31	1.3	5.7	40.8	A/G
31	30	F	A	A	20.98	27	1.1	4.3	46.7	A/G

32	43	F	A	A	21.22	32	0.8	3.8	25.5	A/A
33	30	F	A	A	31.1	24	1.2	3.4	53.3	A/G
34	39	F	A	A	19.33	19	0.7	4.1	21	A/A
35	33	M	A	A	24.58	25	0.6	4.3	63.8	A/A
36	48	F	P	P	29.98	36	1.2	6.1	48.9	A/G
37	39	F	A	A	32.13	26	1.4	5.5	46.3	A/G
38	35	F	A	A	26.47	30	1	4.3	44.3	A/G
39	37	F	A	A	29.14	16	0.8	5.2	27.7	A/A
40	31	F	A	A	20.78	22	0.5	3.3	25.6	A/A
41	35	M	A	A	23.22	18	0.9	4.7	43.4	A/G
42	15	F	A	A	20.01	25	1.2	4.5	42.8	A/G
43	42	M	P	A	25.11	38	1.3	3.6	31.1	A/A
44	37	F	A	A	28.96	33	0.6	5.1	49.8	A/G
45	30	F	A	A	21.11	21	1.1	4.2	72.8	G/G
46	52	F	P	P	33.36	27	1.1	4.9	39.8	A/G
47	27	F	A	A	24.57	36	0.9	3.2	26.7	A/A
48	29	M	A	A	22.78	24	1.2	2.9	81.3	G/G
49	28	F	A	A	28.9	17	1.3	2.1	48.9	A/G
50	36	F	A	A	23.78	28	0.9	4.5	23.5	A/A
51	31	F	A	A	21.93	21	0.6	4.3	52.3	A/G
52	26	F	A	A	34.19	30	0.4	5.1	36.5	A/A
53	38	M	A	A	29.19	27	1	5.4	72.8	G/G
54	29	F	A	A	26.12	19	0.9	6.4	66.5	G/G
55	39	F	A	A	28.23	17	1.3	3.2	22.3	A/A
56	33	F	A	A	29.45	37	1.3	3.3	43.5	A/G
57	41	M	A	A	21.78	23	0.8	5.6	22.3	A/A
58	37	F	A	A	25.09	21	0.5	3.2	53.6	A/G
59	21	F	A	A	23.47	30	0.9	4.5	48.9	A/G
60	23	F	A	A	21.7	48	1.4	8.9	46.6	A/G
61	38	F	A	A	32.79	36	1.1	2.2	27.3	A/A
62	34	F	A	A	18.65	22	0.7	5.5	45.9	A/G
63	46	M	P	A	19.99	26	0.6	5.6	28.6	A/A
64	35	M	A	A	21.78	19	1.1	4.3	25.5	A/A
65	39	F	A	A	34.43	25	0.7	4.8	46.6	A/G
66	50	F	P	A	29.9	30	0.8	2.3	43.3	A/G
67	37	F	A	A	31.78	21	1	6.1	52.3	A/G

68	33	F	A	A	28.7	33	1.1	4.5	52.3	A/G
69	17	M	A	A	23.89	18	0.9	3.9	68.3	G/G
70	35	F	A	A	25.74	27	0.8	4.3	23.6	A/A
71	46	F	P	P	27.02	21	0.8	3.2	45.6	A/G
72	30	F	A	A	27.92	23	1.2	4.4	29.1	A/A
73	37	F	A	A	21.84	39	1.4	5.7	46.3	A/G
74	34	F	A	A	31.92	30	0.6	5.4	52.3	A/G
75	28	M	A	A	26.18	22	0.6	7.2	51.5	A/G
76	31	F	A	A	23.33	17	1.1	4.3	29.8	A/A
77	31	M	A	A	26.77	21	0.9	5.1	26.4	A/A
78	29	F	A	A	25.54	19	0.9	3.8	27.5	A/A
79	35	F	A	A	29.87	25	0.4	3	62.3	A/G
80	41	F	A	A	20.73	23	1.3	4.1	49.8	A/A
81	37	M	A	A	21.01	38	1.2	7.3	45.6	G/G
82	41	F	A	A	28.91	26	0.8	2.1	27.5	A/A
83	30	F	A	A	31.19	33	1	5.9	33.9	A/A
84	34	F	A	A	32.66	25	1.3	3.2	93.8	A/G
85	28	F	A	A	21.49	15	0.9	3.4	47.5	A/A
86	35	F	A	A	27.94	28	1.1	6.2	27.8	A/A
87	32	M	A	A	20.81	30	0.6	5.4	48.9	A/G
88	31	F	A	A	22.94	18	0.4	3.4	18.9	A/A
89	39	F	A	A	23.97	21	0.8	4.5	64.5	A/A
90	48	F	P	A	21.11	39	0.9	5.5	94.8	A/G
91	23	F	A	A	29.84	20	0.5	3.2	18.8	A/G
92	33	M	A	A	31.1	16	1	2.4	49.3	A/G
93	38	F	A	A	28.88	37	1.2	2	56.8	A/G
94	29	F	A	A	19.1	27	0.5	4.5	29.8	A/A
95	36	F	A	A	23.34	31	0.7	3.8	48.5	A/G
96	40	M	A	A	20.17	16	0.7	3.3	36.3	A/G
97	38	F	A	A	32.12	23	1.1	6.4	26.5	A/A
98	39	F	A	A	26.11	36	1	5.4	46.8	A/G
99	28	F	A	A	25.39	27	0.8	3.2	88.9	G/G
100	32	F	A	A	24.1	49	1.5	8.5	28.3	A/A

P-PRESENT A-ABSENT

RESULTS

MASTER CHART 1 & 2:

The Age, Sex, BMI, Serum urea/ creatinine/ uric acid level, Plasma CTLA-4 protein levels and Genotype of 100 SLE cases and control population were tabulated in Master chart 1and 2.

- The Age/sex/BMI of the study population is tabulated to assess the comparability of the two study groups
- Serum urea/ creatinine and uric acid level were analyzed between cases and controls to assess the effect of disease on the blood parameters
- CTLA-4 genotype and CTLA-4 protein levels were assessed between cases and controls, so that it is possible to assess the effect of gene polymorphism over corresponding protein level as well as disease manifestation

COMPARISON OF VARIABLES BETWEEN CASE AND CONTROL POPULATION

The table shows that two groups, cases and controls were age & sex matched, and levels of urea, creatinine and uric acid were significantly higher in SLE patients compared to control population

Table 1

VARIABLE	PATIENTS (N=100)	CONTROLS (N=100)	p-VALUE
AGE	35.57± 7.47	34.83±7.02	0.47- NS
SEX (M/F)	18/82	21/79	0.592- NS
DM/HT (P/A)	17/83	15/85	NS
BMI	25.18± 3.89	25.99± 4.29	0.164-NS
UREA (mg/dL)	45.25±12.92	26.45±7.54	P value <0.001**
CREATININE (mg/dL)	1.2±0.48	0.93±0.27	P value <0.001**
URIC ACID(mg/dL)	6.3±2.13	4.4±1.39	P value <0.001**

M- Male, **F-** Female, **DM-** Diabetes mellitus,

HT- Hypertension, **P-** Present, **A-** Absent

DM (P value = 0.831), HT (P value = 0.70)

TEST FOR HARDY WEINBERG EQUILIBRIUM:

By applying Hardy Weinberg principle, the expected allele frequency is calculated.

Frequency of A allele = 0.54

Frequency of G allele = 0.46

Then expected genotype frequency according to Hardy Weinberg principle is then calculated. Chi square test is performed, using observed genotype frequency and expected frequency calculated by HWE.

Table 2

GENOTYPE	EXPECTED	OBSERVED	p- Value
A/A	58.32	57	Chi square = 0.54 P =0.7
A/G	99.36	102	
G/G	42.32	41	

Statistical Analysis proves that the genotype distribution is in Hardy Weinberg Equilibrium.

DISTRIBUTION OF CTLA-4 GENOTYPE AMONG CASES AND CONTROLS

Table 3

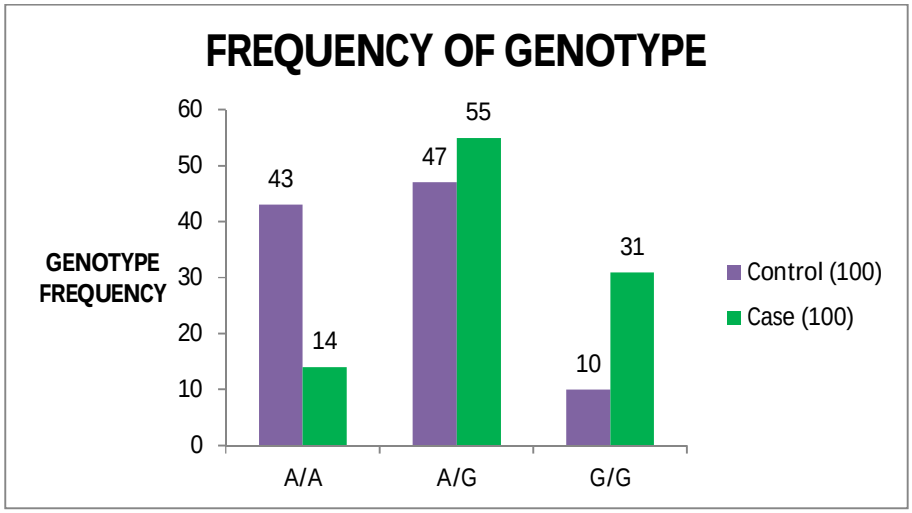
Genotype	Control (100)	Case (100)	P Value
A/A	43	14	Chi square = 26.13 P value <0.001**
A/G	47	55	
G/G	10	31	

The type of CTLA-4 gene among cases and controls was analyzed. The expected frequency of distribution of each of the three genotypes (A/A, A/G, G/G) for cases and controls was then calculated. The significance of difference between the above expected frequency and observed frequency for each of the genotypes was then computed by chi square test.

Chi square – 26.13

P value <0.001**

Statistical analysis proves that there is higher distribution of A/A genotype among healthy population and G/G genotype has higher distribution among SLE patients.



ALLELE DISTRIBUTION AMONG CASES AND CONTROLS

Table 4

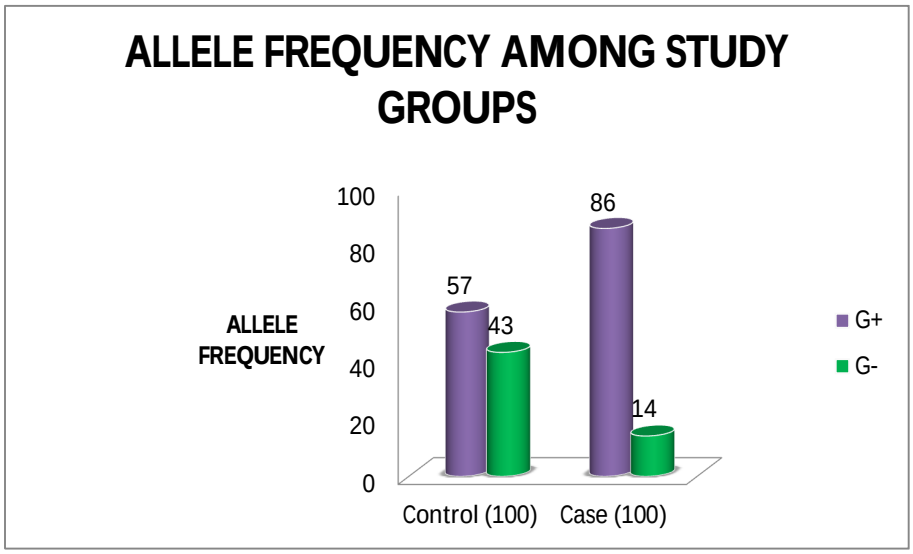
Genotype	Control (100)	Case (100)	P value
G+	57	86	chi square =20.63, P value <0.001**
G-	43	14	

The allele frequency of the cases and controls was computed using allele counting (Observed frequency). The expected frequency of G+ and G- allele for study and control population was then calculated. The significance of difference between expected frequency and observed frequency of alleles among the study population was then computed by chi square test

Chi square = 20.63

P value <0.001**

Statistical analysis proves that there is significantly higher distribution of G allele among cases compared to the control population.



UNIVARIATE ANALYSIS

Table 5

ALLELE	CASES	CONTROLS	Odds ratio
G+	86	57	4.63 (95% CI- 2.33 to 9.23)
G-	14	43	

Univariate analysis is done to assess the risk for the development of SLE if 'G' allele (single variable) is present. Odds ratio which is the measure of exposure and out-come proves that, there is 4.63 times (95% CI – 2.33 to 9.23) increased risk of SLE for the individuals with G allele instead of normal A allele at the 49^{th} position of the exon 1 of CTLA-4 gene

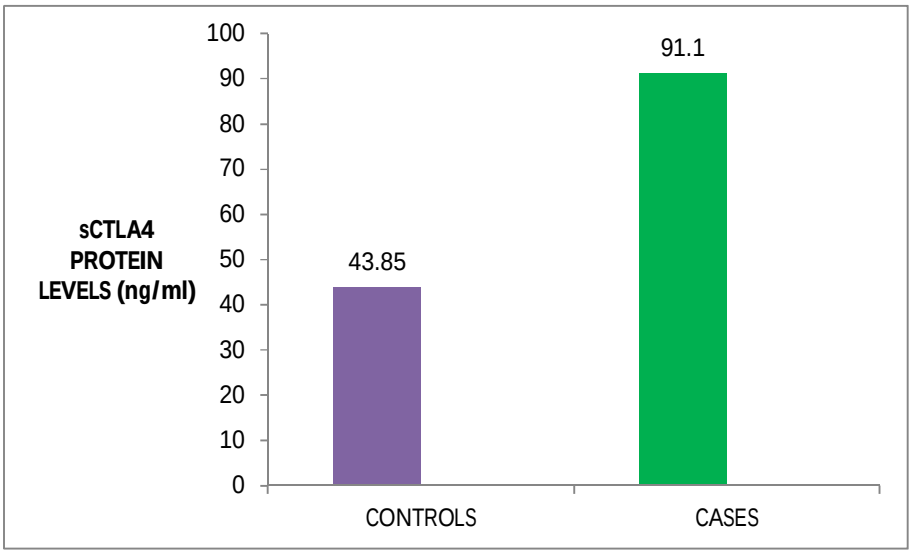
LEVELS OF CTLA-4 PROTEIN BETWEEN CASES AND CONTROLS

Table 6

ANALYTE	CONTROLS	CASES	p-Value
sCTLA-4 protein	43.85±16.90	91.1± 26.45	<0.001**

The level of soluble fragment of CTLA-4 protein in plasma was compared between cases and controls by unpaired t test. There is significantly higher level of sCTLA4 protein levels among cases compared to that of control population (P value <0.001**)

CONCENTRATION OF sCTLA4 PROTEIN

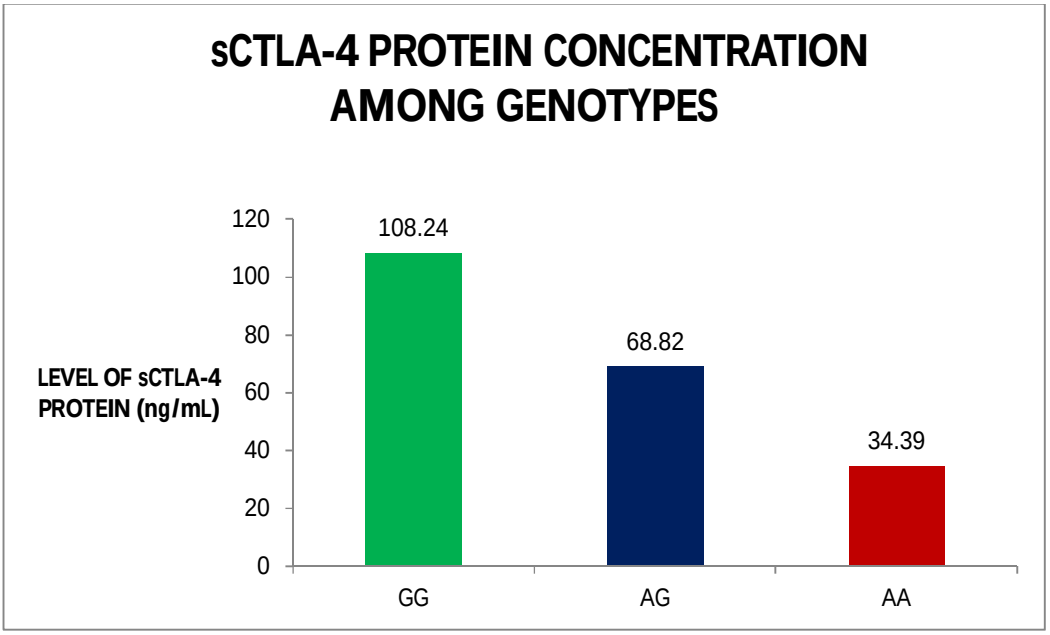


RELATIONSHIP BETWEEN sCTLA-4 PROTEIN LEVELS AND GENOTYPE

Table 7:

GENOTYPE	sCTLA-4 PROTEIN LEVEL (ng/mL)	p-Value
GG	108.24± 25.54	<0.001**
AG	68.82± 19.88	
AA	34.39± 11.14	

There is significantly higher level of sCTLA-4 protein level among individuals with GG genotype compared to those with AA and AG genotype (P value <0.001**)



RELATIONSHIP BETWEEN GENOTYPE AND THE ONSET OF DISEASE:

Table 8:

Genotype	GG	Non-GG	p-VALUE
Age of onset of disease	27.51±4.53	38.9±5.13	<0.001**

The mean age of onset of disease among the individuals with GG genotype is 27.51 years whereas patients with non-GG (A/A &A/G) genotype has mean age of onset of 38.9 years. SLE patients with GG Genotype has comparatively younger age of onset of disease compared to the patients of non-GG (A/A and A/G) genotype. (P value <0.001**)

AGE OF ONSET OF SLE AMONG THE GENOTYPES:

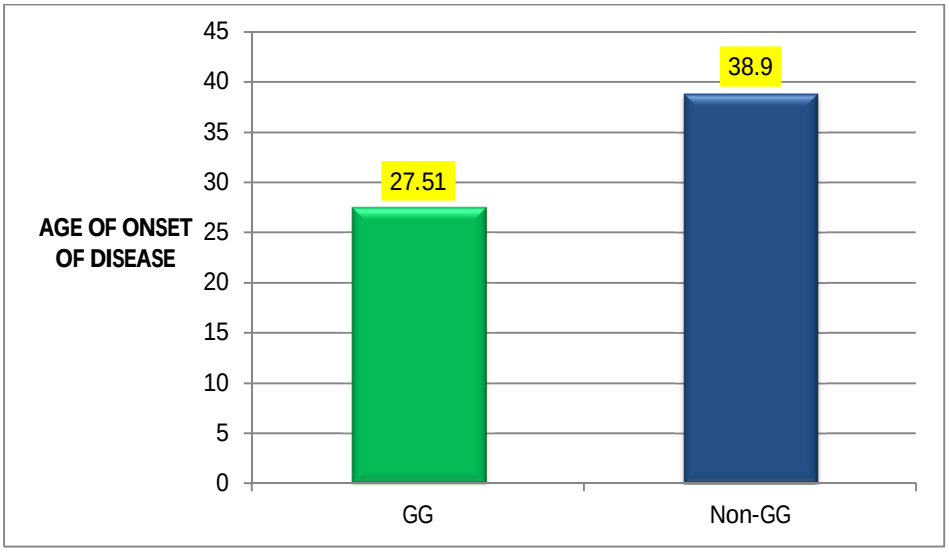


Table 9 : MULTIPLE LOGISTIC REGRESSION ANALYSIS:

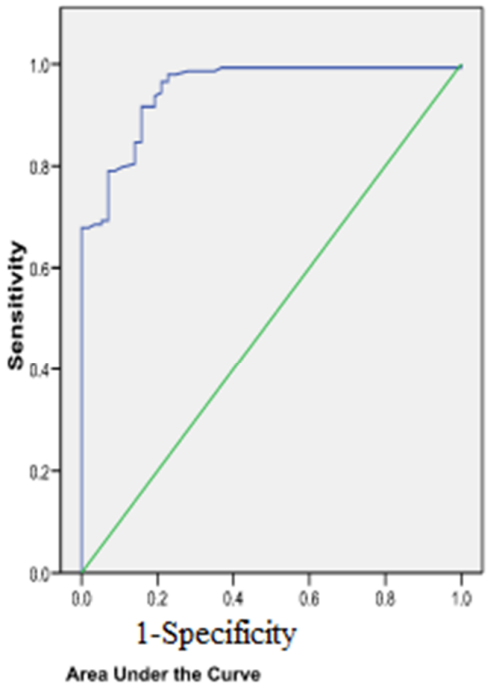
Observed		Predicted		
		Allele		
		G-	G+	Percentage Correct
Allele	G-	4	10	28.6
	G+	2	84	97.7

Table 10

INDEPENDENT VARIABLES	B	S.E	Wald	Df	Sig.	Expo (B)	95% C.I.	
							LOWER	UPPER
AGE	-.153	.068	5.106	1	.024	.858	.751	.980
SEX	-1.13	1.199	.877	1	.349	.325	.031	3.413
DM	.290	1.096	.070	1	.791	1.337	.156	11.450
HT	-1.388	1.511	.844	1	.358	.249	.013	4.823
BMI	.055	.095	.339	1	.560	1.057	.877	1.273
UREA	-.054	.044	1.541	1	.214	.947	.870	1.032
CREATININE	.168	.865	0.38	1	.846	1.183	.217	6.443
URIC ACID	.293	.224	1.707	1	.191	1.340	.864	2.079
CONSTANT	11.060	6.265	3.117	1	.078	63581.128		

Binary Multiple logistic regression analysis is performed. The effect of independent variables on the dependent variable is analyzed. The independent varibles are tabulated above and the dependant variable is presence or absence of the disease. Those with G allele has 97.7% risk compared to those without G allele who have only 28.6% risk. Multiple logistic regression analysis proves the ‘G’ allele is an independent risk factor (except for the age) for the development of the disease. Hence presence of ‘G’ allele is an major risk factor for SLE.

ROC CURVE



VARIABLE : sCTLA4 protein (ng/ml)

Table 11

Area	Standard Error (a)	Asymptomatic Sig. (b)	Asymptomatic 95% Confidence Interval	
.950	.015	.000	.920	.979

The test result variable: CTLA-4 Protein (ng/ml)

The cut- off value of sCTLA-4 protein is 40.4ng/ml. The sensitivity is 97.9% and specificity is 77.2% .

Table 12: Sensitivity and 1-specificity at different concentrations of sCTLA-4 protein in ng/ml

Positive if Greater Than or Equal To(a)	Sensitivity	1 - Specificity
17.800	1.000	1.000
18.850	.993	1.000
19.950	.993	.982
21.650	.993	.965
22.850	.993	.912
23.450	.993	.895
23.550	.993	.877
24.550	.993	.860
25.550	.993	.825
26.000	.993	.807
26.450	.993	.789
26.600	.993	.754
27.000	.993	.737
27.400	.993	.719
27.600	.993	.684
27.750	.993	.667
28.050	.993	.649
28.350	.993	.596
28.500	.993	.579
28.850	.993	.544
29.450	.993	.526
30.050	.993	.474
30.450	.993	.456
30.850	.993	.439
31.450	.993	.421
32.800	.993	.404
33.850	.993	.386
35.100	.993	.368
36.400	.986	.351
37.150	.986	.298
38.800	.986	.281
40.000	.979	.246
40.400	**.979**	**.228**
40.700	.972	.228
41.550	.965	.228
42.550	.965	.211
43.050	.958	.211
43.350	.951	.211

Positive if Greater Than or Equal To(a)	Sensitivity	1 - Specificity
43.450	.944	.211
43.550	.937	.193
43.750	.930	.193
44.100	.923	.193
44.800	.916	.193
45.450	.916	.158
45.700	.902	.158
45.850	.895	.158
46.100	.888	.158
46.450	.874	.158
46.650	.860	.158
46.750	.853	.158
47.150	.846	.158
47.900	.846	.140
48.400	.839	.140
48.700	.832	.140
49.100	.804	.140
49.550	.797	.105
50.500	.790	.088
51.350	.790	.070
51.550	.783	.070
51.950	.776	.070
52.550	.748	.070
53.050	.734	.070
53.350	.727	.070
53.500	.720	.070
54.250	.713	.070
55.600	.706	.070
56.550	.699	.070
57.000	.692	.070
59.750	.692	.053
62.900	.685	.053
63.650	.685	.035
64.150	.678	.018
65.500	.678	.000
66.650	.664	.000
67.550	.657	.000
69.050	.650	.000
71.300	.643	.000
73.000	.629	.000
73.300	.622	.000
73.650	.615	.000

Positive if Greater Than or Equal To(a)	Sensitivity	1 - Specificity
75.200	.608	.000
76.750	.601	.000
77.050	.594	.000
77.700	.580	.000
78.400	.573	.000
78.550	.566	.000
79.200	.559	.000
79.900	.552	.000
80.300	.538	.000
80.950	.531	.000
81.800	.524	.000
82.750	.503	.000
83.300	.490	.000
83.500	.483	.000
83.950	.469	.000
84.600	.462	.000
85.100	.455	.000
85.650	.448	.000
86.100	.441	.000
86.250	.434	.000
86.400	.427	.000
86.550	.420	.000
86.900	.413	.000
87.400	.399	.000
87.900	.392	.000
88.550	.378	.000
89.050	.357	.000
89.250	.343	.000
89.550	.336	.000
89.850	.329	.000
90.350	.322	.000
91.050	.315	.000
91.750	.308	.000
92.250	.301	.000
92.600	.287	.000
93.350	.280	.000
93.850	.273	.000
93.950	.266	.000
94.400	.259	.000
95.400	.252	.000
97.150	.245	.000
98.800	.224	.000

Positive if Greater Than or Equal To(a)	Sensitivity	1 - Specificity
99.550	.217	.000
100.700	.210	.000
102.700	.203	.000
104.700	.196	.000
106.050	.189	.000
106.600	.182	.000
107.250	.175	.000
108.150	.168	.000
108.550	.161	.000
109.150	.154	.000
110.500	.147	.000
112.250	.140	.000
116.050	.133	.000
119.050	.126	.000
119.500	.119	.000
120.050	.112	.000
121.800	.105	.000
123.400	.098	.000
124.250	.091	.000
126.000	.084	.000
127.650	.077	.000
128.800	.063	.000
129.550	.056	.000
130.000	.049	.000
133.200	.042	.000
136.400	.035	.000
139.500	.028	.000

The best cut-off that maximize sensitivity and specificity is 40.4ng/ml.

At this level sensitivity is .979 and specificity is .772 (1-specificity is .228)

DISCUSSION

SLE is associated with a wide range of clinical and biochemical parameters and the disease have a recurrent – remittent course. But, few patients have disease with very severe course and the mortality rate is also very high. Most common causes of death are renal disease, cardiovascular disease and infections. Most SLE patients develop cardiovascular disease at a younger age and develop an attack of MI before the age of 50 years, thus increasing the rate of SLE associated mortality. Despite the invention of many new therapies for the disease and resultant improvement in survival of the patient, the mortality rate for SLE is still high. The increased morbidity/mortality recommends detailed investigations for still unrecognized etiologies (genetic or environmental) that are the putative factors responsible for increasing morbidity and mortality in SLE.

This is a candidate gene study that deals with how variation in CTLA-4 gene results in expression of the disease. The functional relevance of CTLA-4 gene to the disease is that, CTLA-4 gene polymorphism results in uncontrolled proliferation of self-reactive T cells, paving the path for autoimmunity in SLE.

The presence of G allele at 49^{th} position (codon 17) in exon 1 of CTLA 4 gene will code for the aminoacid alanine, whereas A allele at the same locus will code for threonine [156]. Thus polymorphism of the gene results in altered expression of the gene which alters the primary structure of CTLA-4 protein in

the leader peptide sequence. This alteration will then affect the intracellular trafficking of CTLA-4 protein. The surface expression of CTLA-4 protein gets reduced associated with elevated plasma levels of soluble fragment of the protein. The reduced surface expression of the inhibitory protein results in enhanced activity of the T cells against the native antigens.

Statistical analysis proves that A allele is common in healthy population and G allele in those with disease (chi square =20.63, p-Value< 0.001**). The individuals with GG genotype has significantly increased risk of SLE compared AG and AA (Chi square = 26.13, p < 0.001**). There is significantly elevated level of soluble fragment of CTLA-4 protein (p<0.001**) in the plasma of SLE patients compared to that of controls and the individuals with GG genotype have significantly elevated levels of sCTLA4 protein level (p<0.001**) in plasma and compared to AA and GA genotype.

Liu et al[157] studied the expression of CTLA-4 molecules on the surface of activated T cells by flow cytometric analysis and reported that, in polymorphism, although there is normal expression of CTLA-4 protein on the surface of quiescent T cells, there will be weaker expression of CTLA-4 in response to antigenic stimulus thus supporting the present study.

The study by Ueda et al[158] also correlated with the present study. Ueda et al has reported that the common allelic variation is associated with lower mRNA levels of the soluble alternative splice form of CTLA4 in patients with autoimmune disease

The study contrasts with the study by parks et al[159]. Parks et al analyzed all the potential sites of polymorphism in CTLA-4 gene and reported that there is no significant relationship between polymorphism in CTLA-4 gene and occurrence of SLE.

Pullmann et al[160] in 1999 indicated that the "non-MHC linked CTLA-4 gene could confer susceptibility in SLE, as it does in various other autoimmune diseases like Hashimoto thyroiditis, Graves' disease and IDDM." The study done by Ahmed et al[161] in 2001 has concluded that "CTLA-4 gene appears to play a significant role in the development of SLE in the Japanese population". Maurer et al[162] in 2002 has stated that "G allele at position +49 of exon 1 affects the CTLA4-driven down-regulation of T-cell activation and may be an important factor in the pathogenesis of autoimmune diseases". All these studies points strongly towards the correlation between gene polymorphism and SLE.

Previous epidemiological studies have proved that SLE is more common among Asians[163,164] and the clinical severity of the disease will also be worse. So it is crucial to identify the putative risk factor, which make Asians high risk population.

Barreto et al[165], Heward et al[166], Aguliar et al[167] Ulker et al[168] independently investigated the GG genotype distribution among Portugal, England, Spain and Turkish population respectively. The GG genotype distribution among the four populations was around 10% among SLE affected

individuals and 8.5 to8.8% among healthy population. Ahmed et al reported that there was 48% and 31% distribution of GG genotype among SLE patients and healthy controls. The present study identifies 31% distribution of GG genotype among cases and 10% among controls. These data suggest there is higher frequency of GG genotype among Asian population compared to European population, which might be one of the possible reasons that place Asians as high risk population.

Sugimoto et al[169] in 2008 has suggested that GG genotype at 49th position of exon 1 of CTLA-4 gene is associated with earlier onset of SLE in two Japanese families. The present study also correlates with the study by Sugimoto et al. The mean age of onset of SLE in those with non-GG genotype is 38.9 years and those with GG genotype has younger age of onset of disease (mean=27.5 years). Hence the individuals with GG genotype have earlier onset of SLE.

The study proves that individuals with GG and AG genotype have increased risk for SLE, compared to those with AA genotype, confirming the fact that 'G' allele is associated with increased risk for the development of this autoimmune condition. There will be altered expression of the gene associated with polymorphism, altering the intracellular trafficking. The reduced expression of CTLA-4 protein on the surface of T cells result in reduced negative regulation on T cells, resulting T cells becomes active against self-antigens.

CONCLUSION

The conclusions arrived by the present study are

1. **CTLA-4 gene polymorphism (+49 A/G) is associated with the development of SLE**.

   CTlA-4 gene has 4 exons, the polymorphism in exon 1 of the gene at 49^{th} position in which A allele if replaced by G allele will increase the risk of SLE. There is higher distribution of G/G and A/G genotype among cases compared to controls.

2. **G allele in fact is the independent risk factor for the development of disease**

   The individuals with risk allele (G allele) have 4.6 times increased risk of SLE.

3. **There will be elevated level of soluble fragment of CTLA-4 protein in plasma of SLE patients** and those with risk allele (G allele) have higher level of sCTLA-4 protein compared to those with non-risk allele (A allele).

4. **The patients with GG genotype has comparatively younger age of onset of SLE, than those of non-GG SLE patients.**

LIMITATIONS OF THE STUDY

- ✓ SLE is a multifactorial disease that multiple genetic factors along with environmental exposure makes the person susceptible prey for this autoimmune condition. The study analyzes the effect only a single genetic variation that accounts only a smaller percentage to the occurrence of disease. **The study does not take into account other genetic alterations and environmental factors that in combination leads to the occurrence of the disease.**

- ✓ SNP study has multiple implications.
    1. SNP helps in diagnosis of a clinical condition
    2. SNP studies help to assess the severity and prognosis of the disease among individual patients
    3. The response of the patients to therapy can be assessed by studying the genetic variation among individuals

The study takes into account only the effect of gene polymorphism on the occurrence of disease. **The severity and prognosis of the disease in polymorphism and the response of individuals with polymorphism to drug therapy are not scope of the current study.**

FUTURE SCOPE OF THE STUDY

- Identification of **SNP in CTLA4 gene among families**, contributes to the earlier identification of susceptible individuals to SLE. Regular monitoring of those susceptible individuals helps in earlier diagnosis as well as earlier therapy so as to prevent later complications.
- The study proves that **soluble fragment of CTLA-4 protein is elevated in SLE patients** irrespective of disease activity, hence
    a) sCTLA-4 protein can be used as novel surrogate marker of SLE
    b) Antibodies to sCTLA-4 protein can be used as therapy for SLE
- The strength of association between **CTLA-4 gene polymorphism and severity & prognosis of the disease** can be assessed
- The effective response of the patients with CTLA-4 gene polymorphism to therapy and effect of therapy on the levels of sCTLA-4 protein can be assessed.

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INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE, CHENNAI 600 003

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CERTIFICATE OF APPROVAL

To

Dr.M.Divya
II Year PG in MD (Bio-Chemistry)
Institute of Bio-Chemistry
Madras Medical College
Chennai 600 003

Dear Dr.M.Divya,

The Institutional Ethics Committee has considered your request and approved your study titled **"THE SIGNIFICANCE OF CTLA 4 (CYTOTOXIC T LYMPHOCYTE ASSOCIATED PROTEIN 4) GENE POLYMORPHISM IN SYSTEMIC LUPUS ERYTHEMATOSUS "** NO.14012015.

The following members of Ethics Committee were present in the meeting hold on 20.01.2015 conducted at Madras Medical College, Chennai 3.

1. Dr.C.Rajendran, MD	:Chairperson
2. Dr.R.Vimala,MD.,Dean,MMC,Ch-3	: Deputy Chairperson
3. Prof.B.Kalaiselvi,MD.,Vice Principal,MMC,Ch-3	: Member Secretary
4. Prof.R.Nandhini,MD.,Inst.of Pharmacology,MMC	: Member
5. Prof.P.Ragumani, MS., Professor, Inst.of Surgery,MMC	: Member
6. Prof.K.Ramadevi, Director , Inst.of Bio-Chem.MMC	: Member
7. Prof.Saraswathy,MD.,Director,Pathology, MMC	: Member
8. Prof.Md.Ali, MD., DM.,Prof.&HOD of Medl.GE,MD.MMC	: Member
9. Thiru S.Rameshkumar	: Lay Person
10.Thiru S.Govindasamy, BA., BL.,	: Lawyer
11.Tmt.Arnold Saulina, MA., MSW.,	: Social Scientist

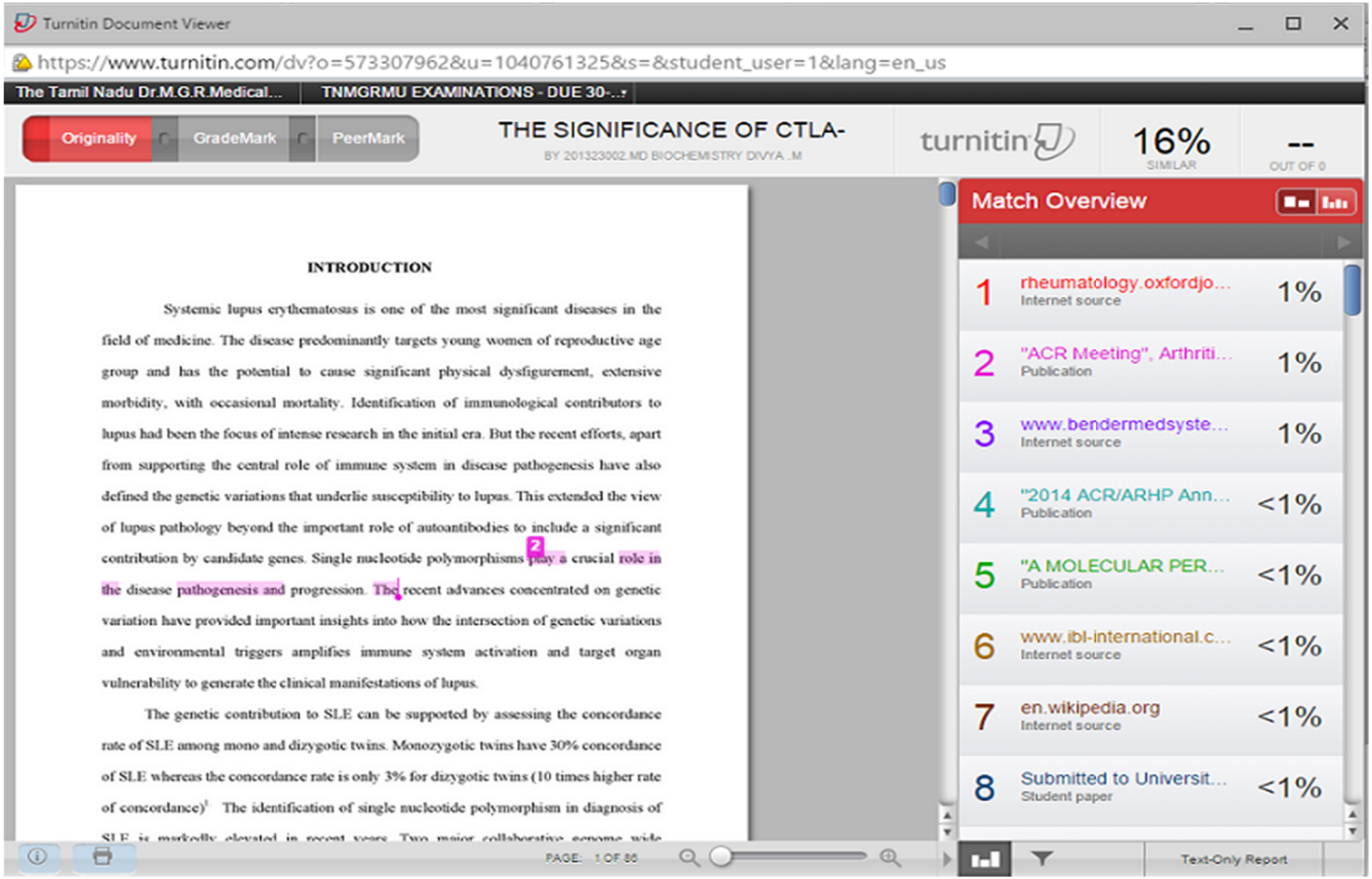
We approve the proposal to be conducted in its presented form.

The Institutional Ethics Committee expects to be informed about the progress of the study and SAE occurring in the course of the study, any changes in the protocol and patients information/informed consent and asks to be provided a copy of the final report.

Sys 2

Member Secretary – Ethics Committee

MEMBER SECRETARY
INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE
CHENNAI-600 003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INTRODUCTION
Systemic lupus erythematosus is one of the most significant diseases in the field of medicine. The disease predominantly targets young women of reproductive age group and has the potential to cause significant physical dysfigurement, extensive morbidity, with occasional mortality. Identification of immunological contributors to lupus had been the focus of intense research in the initial era. But the recent efforts, apart from supporting the central role of immune system in disease pathogenesis have also defined the genetic variations that underlie susceptibility to lupus. This extended the view of lupus pathology beyond the important role of autoantibodies to include a significant contribution by candidate genes. Single nucleotide polymorphisms play a crucial role in the disease pathogenesis and progression. The recent advances concentrated on genetic variation have provided important insights into how the intersection of genetic variations and environmental triggers amplifies immune system activation and target organ vulnerability to generate the clinical manifestations of lupus.
The genetic contribution to SLE can be supported by assessing the concordance rate of SLE among mono and dizygotic twins. Monozygotic twins have 30% concordance of SLE whereas the concordance rate is only 3% for dizygotic twins (10 times higher rate of concordance)
The identification of single nucleotide polymorphism in diagnosis of
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INFORMATION SHEET

Title of the study : **THE SIGNIFICANCE OF CTLA-4(CYTOTOXIC T-LYMPHOCYTE ASSOCIATED PROTEIN 4) GENE POLYMORPHISM IN SUSCEPTIBILITY TO SYSTEMIC LUPUS ERYTHEMATOSUS**

SLE is a systemic autoimmune disease in which immune system will destroy body's own cells and tissues leading to tissue damage. The peak age of onset is between 20-40 years. The aetiology of is SLE unknown and recent reports states that there is genetic susceptibility to SLE and CTLA-4 gene polymorphism is associated with increased susceptibility to SLE.

The purpose of this study is to assess the significance of ctla-4 gene polymorphism in susceptibility to systemic lupus erythematosus in Rajiv Gandhi Govt. General Hopital Chennai. To do this study, I need to collect 5 ml of blood ,there will not be any side effects.

Your identity will be confidential throughout the study and during publication and presentation in any journal and clinical forums. Participation in this study is purely voluntary. You can withdraw from this study at any time. Your decision will not result in any loss of benefits to which you are otherwise entitled. The results of the study will be intimated to you. If you have willingness to participate in this study, kindly sign in this information sheet and consent form.

Signature of investigator Signature of participant

Place:

Date:

PATIENT CONSENT FORM

Title of the study: **THE SIGNIFICANCE OF CTLA-4(CYTOTOXIC T-LYMPHOCYTE ASSOCIATED PROTEIN 4) GENE POLYMORPHISM IN SUSCEPTIBILITY TO SYSTEMIC LUPUS ERYTHEMATOSUS**

Documentation of the informed consent

I_____ have read the information in this form (or it has been read to me). I was free to ask any questions and they have been answered. I hereby give my consent to be included as a participant in the study: **THE SIGNIFICANCE OF CTLA-4 GENE POLYMORPHISM IN SUSCEPTIBILITY TO SYSTEMIC LUPUS ERYTHEMATOSUS.**

- I have read and understood this consent form and information provided to me.
- I have had the consent document explained to me.
- I have been explained about the nature of the study.
- I have been explained about my rights and responsibilities by the investigator.
- I have been informed the investigator of all the treatments I am taking or Have taken in the past months including native(alterative) treatment.
- I have been advised about the risks associated with my participation in this study.
- I agree to cooperate with the investigator and I will inform him/her immediately if I suffer unusual symptoms.
- I have not participated in any research study within the past_____months.
- I am aware of the fact that I can opt out of the study at any time without having to give reason and this willnot affect my future treatment in this hospital.
- I am also aware that investigator may terminate my participation in the study at any time, for any reason, without any consent.
- I hereby give permission to the investigators to release the information obtained from me as result or participation in this study to the sponsers , regulatory authorities , Govt. agencies and IEC. I understand that they are publicly presented.
- I have understand that my identity will be kept confidential if my data are publicly presented.
- I have had my questions answered to my satisfaction.

- I have decided to be in the research study.

I am aware that if I have any questions during this study, I should contact the investigator. By signing this consent form I attest that information given in this document has been clearly explained to me and understood by me, I will be given a copy of this consent document.

For participants:

Name and signature of the impartial witness(required for illiterate patients)
Name_____Signature_____Date_____

Address and contact number of the impartial witness:

Name and signature of the investigator or his representative obtaining consent:
Name_____Signature_____Date_____

சுய ஒப்புதல் கடிதம்

ஆராய்ச்சி தலைப்பு: உள்பரவிய செம்முருடு நோய் தாக்கபட்டவர்களில் சி.டி.எல்.ஏ 4 மரபணு பாலிமார்ஃபிஸத்தின் பங்கு

பங்கேற்பவரின் பெயர் :

பங்கேற்பவரின் எண்:

- மேலே குறிப்பிட்டுள்ள மருத்துவ ஆய்வின் விவரங்கள் எனக்கு தெளிவாக விளக்கப்பட்டது.என்னுடைய சந்தேகங்களை கேட்கவும், அதற்கான தகுந்த விளக்கங்களை பெறவும் வாய்பளிக்கப்பட்டது.

- நான் இந்த ஆய்வில் தன்னிச்சையாக பங்கேற்கிறேன். எந்த கட்டத்திலும் நான் இந்த ஆய்வில் இருந்து விலகி கொள்ளலாம் என்றும் அறிந்து கொண்டேன்,

- இந்த ஆய்வின் மூலம் கிடைக்கும் தகவல்களையும், பரிசோதனை முடிவுகளையும் மற்றும் சிகிச்சை தொடர்பான தகவல்களையும் மருத்துவர் மேற்கொள்ளும் ஆய்வில் பயன்படுத்தி கொள்ளவும் அதை பிரசுரிக்கவும் என் முழு மனதுடன் சம்மதிக்கிறேன்

- இந்த ஆய்வில் எனக்கு இரத்தப் பரிசோதனை மற்றும் சிறுநீரக பரிசோதனை செய்துகொள்ள முழு மனதுடன் சம்மதிக்கிறேன்

- இதன் மூலம் எந்த பின்விளைவும் வராது என மருத்துவர் மூலம் தெரிந்து கொண்டு என்னுடைய சுயநினைவுடன் மற்றும் முழு சுதந்திரத்துடன் இந்த மருத்துவ ஆராய்ச்சியில் என்னை சேர்த்துக்கொள்ள சம்மதிக்கிறேன் .

பங்கேற்பாளர் கையொப்பம் .....இடம்.....தேதி

கட்டைவிரல் ரேகை

பங்கேற்பாளர் பெயர் மற்றும் விலாசம்.....

ஆராய்ச்சியாளர் கையொப்பம் .....இடம்.....தேதி

ஆராய்ச்சி தகவல் தாள்

இராஜீவ் காந்தி அரசு பொது மருத்துவமனைக்கு வரும் உள்பரவிய செம்முருடு நோயாளிகளுக்கு சி.டி.எல்.ஏ 4 மரபணு பாலிமார்ஃபிஸத்தின் முக்கியத்துவத்தை கண்டறிதலே இந்த ஆராய்ச்சியின் நோக்கமாகும்

இந்த ஆராய்ச்சியில் உங்களுடைய இரத்தம் மற்றும் சிறுநீர் சில சிறப்புப் பரிசோதனைகளுக்கு உட்படுத்தப்படும். அதனால் தங்களது நோயின் ஆய்வறிக்கையோ அல்லது சிகிச்சையோ பாதிப்புக்கு உள்ளாகாது என்பதையும் தெரிவித்துக் கொள்கிறோம்.

இந்த ஆராய்ச்சியின் முடிவுகளை அல்லது கருத்துக்களை வெளியிடும் போதோ அல்லது ஆராய்ச்சியின் போதோ தங்களது பெயரையோ அல்லது அடையாளங்களையோ வெளியிடமாட்டோம் என்பதையும் தெரிவித்துக் கொள்கிறோம்.

இந்த ஆராய்ச்சியில் பங்கேற்பது தங்களுடைய விருப்பத்தின் பேரில் தான் இருக்கிறது.மேலும் நீங்கள் எந்நேரமும் இந்த ஆராய்ச்சியிலிருந்து பின்வாங்கலாம் என்பதையும் தெரிவித்துகொள்கிறோம்.

இந்த சிறப்பு பரிசோதனைகளின் முடிவுகளை ஆராய்ச்சியின் போது அல்லதுஆராய்ச்சியின் முடிவின் போது தங்களுக்கு அறிவிப்போம் என்பதையும் தெரிவித்துக்கொள்கிறோம்

ஆராய்ச்சியாளர் கையொப்பம் பங்கேற்பாளர் கையொப்பம்

தேதி:

CLINICAL PROFORMA

PATIENT DETAILS:

Name: Age: Sex:

Occupation:

Address:

Phone number:

Hospital number:

COMPLAINTS:

H/O **S**kin lesions

H/O Photosensitivity

H/O Oral ulcers

H/O Joint pain

H/O chest pain/dyspnea

H/O facial puffiness/swelling of legs

H/O Seizures

H/O altered behavior

Relevant past History:

Family History:

Treatment History:

CLINICAL EXAMINATION:

Pulse BP:

RR:

GENERAL EXAMINATION:

CVS:

RS:

P/A:

CNS:

INVESTIGATIONS:

- Blood Urea
- Serum Creatinine
- Serum Uric Acid
- Assay of sCTLA-4 protein
- CTLA-4 gene polymorphism analysis.